Investigation of chemical components and pharmaceutical potential of *Carpobrotus* species

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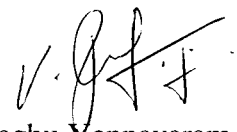

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Dedication

To my Mom and all people close to my heart.....

Abstract

Objectives: To investigate the chemistry and pharmaceutical potential of three *Carpobrotus* species found in Tasmania, *C. edulis*, *C. rossii* and *C. aequilaterus*.

Methods: The plant juice and methanolic extracts of plants were analysed for chemical constituents by LC-MS, GC-MS, HPLC-DAD, HPLC-ELSD, flame photometry, elemental analysis, acid titration and gravimetric analysis. Based on folklore use, a gel formulation containing plant juice of *C. rossii* was used in both a placebo controlled double-blinded clinical trial to investigate its potential as a common hand wart treatment, and in a histamine skin prick model to investigate topical anti-inflammatory activity. The antioxidant activity of the plant juice of the three species was also measured by the DPPH, FRAP and β-carotene assays, and the contribution of ascorbic acid was investigated. The antioxidant activity was isolated in a single fraction using Sephadex LH-20 gel and the fraction was analysed by LC-MS.

Results: Both plant juice and methanolic extract contained flavonoids, tannins and carbohydrates. The plant juice contained, in addition, inorganic salts, titratable acids and volatile constituents that were primarily grassy aldehydes. Novel flavonoids whose structures have yet to be elucidated were present in the *C. rossii*. The anti-wart trial was conducted over an 8 week period and recruited a total of 50 subjects of whom 41 subjects (19 cases, 22 controls) completed the study. The histamine skin prick model was used in 12 subjects. Both the anti-wart trial and the histamine skin prick model failed to show any significant effects of *C. rossii* extract. The plant juices from *C. edulis*, *C. rossii* and *C. aequilaterus* were all found to have very strong antioxidant activity with typical levels equivalent to around 13 mg/ml of rutin, 2.5 mg/ml of gallic acid or 9 mg/ml of ascorbic acid. Ascorbic acid levels could

account for about 10% of antioxidant activity. Antioxidant activity guided fractionation by gel filtration column chromatography and analysis of this fraction by LC-MS showed the presence of condensed tannins as major chemical constituents, which appeared to account for the majority of the antioxidant activity. LC-MS analysis of *C. rossii* showed the presence of flavonoids that contained a substituent consistent with the rarely encountered 3-hydroxy-3-methylglutaric acid moiety.

Conclusion: *C. rossii* extract gel is not effective in the treatment of common warts and does not possess topical anti-inflammatory action against histamine. There is significant antioxidant activity present in *Carpobrotus* species. Further investigation of the antioxidant compounds and their potential therapeutic applications are warranted. Further screening for other biological activities, particularly of the novel flavonoids, is also recommended.

List of abbreviations

AAC	Antioxidant activity coefficient
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
APF	Australian Pharmaceutical Formulary and Handbook
ATP	Adenosine triphosphate
BHA	Butylated hydroxylanisole
BHT	Butylated hydroxytoluene
C. aequilaterus	*Carpobrotus aequilaterus*
C. chilensis	*Carpobrotus chilensis*
C. edulis	*Carpobrotus edulis*
C. muirii	*Carpobrotus muirii*
C. quadrifidus	*Carpobrotus quadrifidus*
C. rossii	*Carpobrotus rossii*
DCM	Dichlorometheylene
DPPH	2,2-diphenyl-2-picrylhydrazyl hydrate
ELSD	Evaporative light scattering detector
ESI	Electrospray ionisation
Fe^{+2}	Ferrous ion
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferrous sulphate
FRAP	Ferric reducing and antioxidant power
GAE	Gallic acid equivalents

GC-MS	Gas chromatography with mass spectrometry
HCL	Hydrochloric acid
HPLC	High performance liquid chromatograph
HPLC-DAD	High performance liquid chromatography with diode array detection.
KCl	Potassium chloride
K^+	Potassium ion
LC-MS	Liquid chromatography and mass spectrometry
NaCl	Sodium chloride
Na^+	Sodium ion
PDA	Photodiodearray
SPME	Solid Phase Micro Extraction
TPTZ	2,4,6-tripyridyl-s-triazine
UV	Ultra-violet

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Chapter 1: Introduction

1.1 Objective of the study

The major aims of this study were to investigate the chemical composition and pharmaceutical potential of the plants *Carpobrotus aequilaterus, C. edulis* and *C. rossii,* (Aizoaceae) commonly called pig face. Objectives included a detailed assessment of the antioxidant activity of the plants and investigation of a formulation containing *C. rossii* constituents in an anti-inflammatory histamine skin prick model and as an anti-wart treatment.

1.1.1 Background

The World Health Organisation estimates that 80% of the people living in developing countries almost exclusively use traditional medicines. Medicinal plants form the principal components of traditional medicine. In 1984, at least 25% of the prescription drugs issued in the USA and Canada were derived from or modelled after plant natural products (Farnsworth, 1984).

The number of resistant strains of microbial pathogens has grown since penicillin-resistant and multi-resistant pneumococci caused a major problem in South African hospitals in 1977. Berkowitz called the emergence of drug resistant bacteria a "medical catastrophe" (Berkowitz, 1995). Leggiadro (1995) stated that effective regimes might not be available to treat some enterococcal isolates and that it is critically important to develop new anti-microbial compounds for these and other

organisms. Compounds inhibiting microorganisms, such as emetine and benzoin, have been isolated from plants (Cox, 1994). Anti-microbial compounds from plants may inhibit bacteria by a different mechanism from presently used antibiotics and may have clinical value in treatment of resistant microbial strains (Eloff, 1998). The development of new antibiotics, either those that block or circumvent resistance mechanism or those that attack new targets are essential. A different approach is to focus on preventing infection by inhibiting key gene products that are involved in the infection process itself (Levy and Marshall, 2004).

1.2 *Carpobrotus*

Carpobrotus N. E. Br., Gard. Chron. Ser. 3, 78: 433 (1925); from the Greek *karpos* (fruit), and *brota* (edible thing), referring to the edible fruits (Venning, 1984). The genus *Carpobrotus* belongs to the family Aizoaceae. *Carpobrotus* species are large, trailing, leaf succulent perennials. They have long stems that root at the nodes, with triangular cross section leaves. Leaves are sometimes tinged with red, depending on the growing conditions. *Carpobrotus* species grow on the Pacific coast of the Americas, in South Africa and southern Australia. Most of the species grow in the coastal regions, and also in sandy areas. They can be useful to hold drifting sand, and to colonize rapidly disturbed areas where water is of limited availability. Most of the species bloom in early summer and will take some frost (Venning, 1984).

The most widespread species of *Carpobrotus* in Australia are *C. edulis, C. glaucescens, C. aequilaterus* and *C. rossii. C. edulis* is native to South Africa but has naturalised in Australia in every state. The local Tasmanian species is *C. rossii*, called

"pig face" by the locals. *C. rossii* is a common species and is widespread in coastal areas. It is believed that Tasmanian Aboriginals used this plant for its medicinal value. Although the plant may have been used for its antimicrobial activities there is a paucity of scientific information to support its use.

1.2.1 *Carpobrotus rossii*

The name of the species *Carpobrotus rossii* was coined after the botanical collector William Ross. *C. rossii* (Figure 1.1) is a fleshy perennial plant with prostrate stems up to 1 m long. Leaves are triangular in section; opposite bases stem-clasping; tips pointed. These act as the water storage organs enabling the plant to survive hot dry summers on coastal cliffs and sand. *C. rossii* has light purple flowers 4-6 cm in diameter, solitary on the ends of short lateral branches, with a fleshy calyx and many shining narrow strap-shaped petals about 2 cm long in several rows. There are many stamens and white filaments. Fruits are fleshy, fig-like, with small seeds (Venning, 1984).

Figure 1.1 *Carpobrotus rossii* growing on a typical coastal site.

1.2.2 *Carpobrotus aequilaterus*

Carpobrotus aequilaterus (Figure 1.2) is also referred to as *C. chilensis*. *C. aequilaterus* is an evergreen perennial growing to 0.2 m height. The plant prefers light (sandy) and medium (loamy) soils, requires well drained soils and can grow well in nutritionally poor soils. It can not grow in shade but is able to grow in acidic, basic, neutral as well as saline soils. It can also tolerate drought. Flowers are purple, lighter towards the base and 6.5-8 cm in diameter. Stems are biangular, 8-10 mm in diameter. Leaves are denticulate in their upper part and entire below. Fruits are fleshy and indehiscent. *C. aequilaterus* is introduced to North America confined mostly to the southern parts including California and Mexico, and to Australia. It is found in New South Wales, Tasmania and Victoria (Venning, 1984).

Figure 1.2 *Carpobrotus aequilaterus* plant with its flower.

1.2.3 *Carpobrotus edulis*

Carpobrotus edulis (Figure 1.3) is a perennial succulent from South Africa, which has been introduced on the coasts of southern and western Europe including the United Kingdom and Republic of Ireland. It has also been introduced to Australia.

C. edulis plants have triangular, fleshy, grey-green leaves, which are slightly curved and tapering at the ends and which grow on woody prostrate stems. They bloom daisy-like yellow flowers up to 12.5 cm across, that open after noon and turn pinkish later in the day. The flowering season is from early spring to summer. They bear a fig-like brown fruit that is edible and can be used as a preserve. The leaves are also edible and taste like pickled cucumber. It is called "sour fig" in South Africa (Venning, 1984). *C. edulis* inhabits well-drained soils and rocky places like cliffs. It is used to bind sandy soil (Venning, 1984).

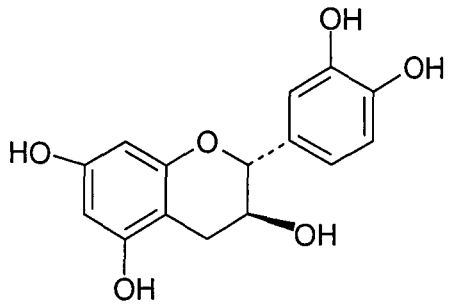
Figure 1.3 *Carpobrotus edulis* plant with flower.

C. edulis has been claimed to possess many medicinal uses, but these uses have yet to be proven in clinical studies. Major actions attributed to the plant can be broadly grouped into antibacterial, antifungal and antiseptic properties. The leaf juice is

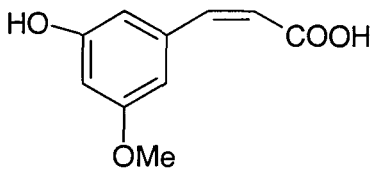
antiseptic and is traditionally used as a gargle to treat mouth and throat infections. It is used as a traditional remedy for a wide range of fungal and bacterial infections (Smith *et al.*, 1998). The juice is also taken orally as a remedy for dysentery, digestive troubles, sinusitis and tuberculosis, and used for other internal chest problems (van Wyk, 1997). The leaf juice is reportedly effective in soothing itching caused by spider and tick bites (Roberts, 1990). It is highly astringent and used externally on wounds, burns and to treat eczema. It is also thought to be effective against toothache, earache and oral and vaginal thrush.

Other *Carpobrotus* species have been analysed for antimicrobial activities. South African species *C. muirii* and *C. quadrifidus* showed antimicrobial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* (Springfield *et al.*, 2003). *C. edulis* methanolic extract inhibited the growth of phagocytosed multi drug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (Martins *et al.*, 2005).

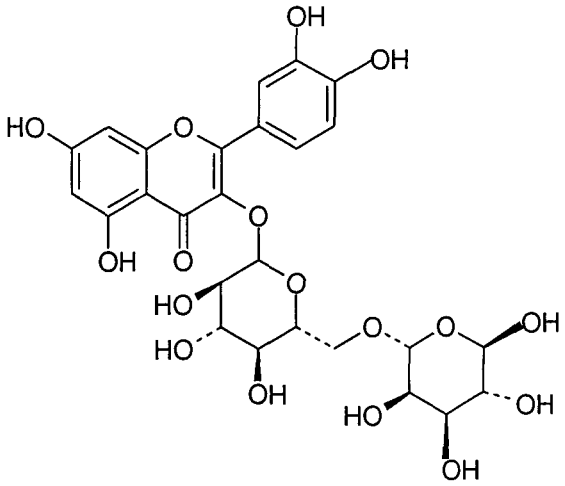
There are few studies describing biologically active metabolites from other genera of the Aizoaceae family. Polysaccharides isolated from *Tetragonia tetragonoides* had anti-inflammatory activity against carrageenin-induced edema in rats (Koho, 1984). An antifungal tetraterpenoid named trianthenol was isolated from the chloroform extract of *Trianthema portulacastrum* (Nawaz, *et al.*, 2001).



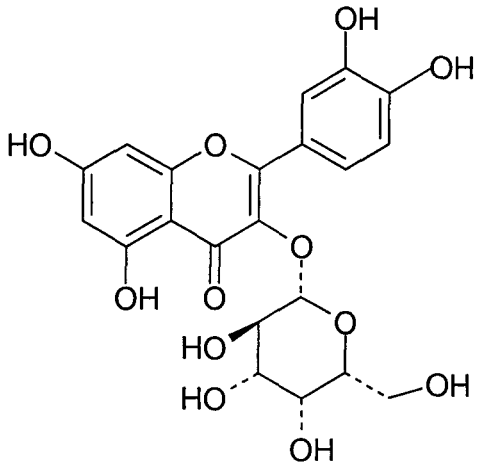
Catechin



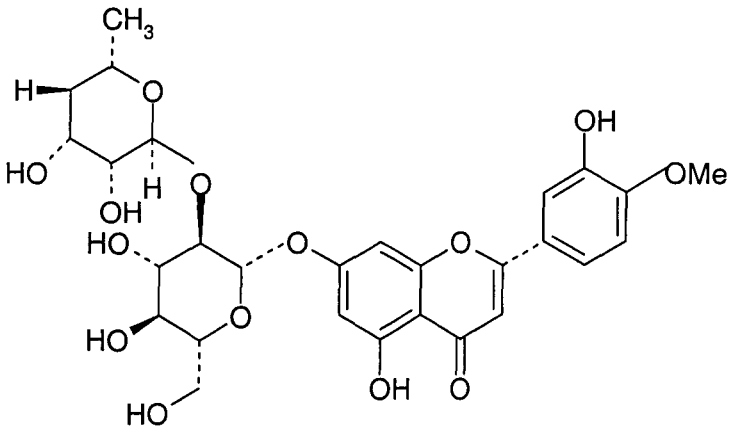
Ferulic acid



Rutin



Hyperoside



Neohesperidin

Figure 1.4 Chemical structures of the main antibacterial chemical constituents of *C. edulis*.

1.2.4 Major chemical constituents of *Carpobrotus edulis*

There has been only one published study of the chemical constituents of *C. edulis* (van der Watt and Pretorious, 2001). The key chemical components present are mainly polyphenolics. Flavonoids isolated from *C. edulis* have been found to possess antibacterial activity and are shown in Figure 1.4.

Antibacterial activity was qualitatively evaluated by the agar plate diffusion assay technique. Three positive controls (10 μg ampicillin, 30 μg chloramphenicol and 10 units penicillin) were used. All five identified and one unidentified compounds had antibacterial activity against *Moraxella catharralis* (gram negative), *Staphylococcus epidermidis* and *S. aureus* (gram positive). Neohesperidin and hyperoside were active against *Pseudomonas aeruginosa* (van der Watt and Pretorious, 2001).

1.3 Flavonoids

Plants produce a high range of diversity in secondary metabolites for defence and survival in the ecosystem. Secondary plant metabolites usually belong to three major chemical classes; terpenes, phenolics and alkaloids. Secondary metabolites are sought after because they have numerous biological activities with possible positive therapeutic effect (Oomah, 2003). It is estimated that flavonoids and related compounds, form about 2% of all carbon photosynthesized by plants (Markham, 1982).

Flavonoids are phenolic secondary metabolites of plants, which are secreted and stored in different parts of the plant. Flavonoids include the major colouring

principles in plants. They also act as enzyme inhibitors, protect plants from the damage of ultraviolet radiation exposure and chelate noxious metals (Cuyckens and Claeys, 2004). Flavonoid aglycones are polyphenols and as such possess the chemical properties of phenolics. They are slightly acidic in nature and will dissolve in alkali.

1.3.1 Occurrence

Flavonoid compounds usually occur in all parts of the higher plants; in roots, leaves, flowers, fruits, seeds, pollen, wood and bark. Usually, different classes of flavonoids are more characteristic to some particular tissue than others. Anthocyanins are pigments present mostly in fruits, flowers and leaves. They also occur in other parts of plants, but they are confined to or occur in higher concentrations in one kind of tissue. Chalcones and aurones are largely found in flower petals. Catechins and flavan-3,4-diols are mostly present in wood and bark. Flavones, flavonols and flavonones occur in many parts of plants and are not characteristic or confined to any particular kind of plant tissue (Geissman, 1962).

1.3.2 Pharmacological activity of flavonoids

The pharmacological actions of the flavonoids are usually weak when compared with other plant secondary metabolites. The vast majorities of flavonoids are non-toxic to man and are widely distributed in foods. Rutin and the related flavanones, hesperidin and eriodictyol, have been proposed to decrease the fragility of blood capillaries in guinea pigs and were considered to possess vitamin-like activity in humans (Harborne, 1967). Rutin is widely used in multivitamin tablets. It also is believed to have efficacy in treatment of blood pressure, relaxes smooth muscles, behaves as a

general enzyme inhibitor and has pharmacological activity as an antioxidant towards adrenaline and ascorbic acid (Harborne, 1967). The isoflavones, genistein and daidzein, were found to be oestrogenic, after isolation from Australian strains of subterranean clover. These isoflavones are pro-oestrogens and are converted to active substances in the body during their metabolism (Harborne, 1967).

1.3.3 Flavonoids in food products

Flavonoids are widely distributed in foods. Anthocyanins are present in major fruits and vegetables; apples, cherries, grapes, cabbages, onions, oranges, pears, plums, potatoes, raspberries, peaches, gooseberries, radishes and pomegranates. Flavonoids that belong to other classes are also present, such as leucoanthocyanidins and catechins, which give flavour and provide astringency. Catechins occur in tea leaves (Harborne, 1967).

1.4 Isolation and separation

The main aim and focus in analysis of natural products is the characterisation of chemical constituents with the minimum amount of sample preparation. The main process by which separation of compounds can be achieved rapidly and routinely is by chromatography. There are many chromatographic techniques, but all of them have in common separation of compounds through the use of variations in mobile and stationary phases. Liquid chromatography (LC) and gas chromatography (GC) are used online with mass spectrometry, which is a useful method of identifying unknown compounds. Gas chromatography is widely used for its excellent selectivity and sensitivity, but the basic requirement of gas chromatography is that the sample should

be thermally stable with appreciable vapor pressure at a temperature within the operating range of the column. Liquid chromatography is primarily used for the separation of high molecular weight, non-volatile and polar compounds in one of two phases, that is normal phase, and reverse phase chromatography. Reversed phase chromatography is the most widely used separation mode (Oomah, 2003).

1.5 Liquid chromatography and mass spectrometry (LC-MS)

The most efficient and powerful tool for the analysis of natural products is by coupling liquid chromatography with mass spectrometry (LC-MS). Flavonoid analysis is not usually done by gas chromatography with mass spectrometry (GC-MS), because flavonoid glycosides are insufficiently volatile. Derivatisation can be useful, but is time consuming and fragmentation patterns of derivatives are often difficult to interpret. Mass spectrometry is highly sensitive and provides information on structure and molecular mass (Cuyckens and Claeys, 2004). Information regarding the structure of flavonoids can be obtained on the aglycone moiety, types of carbohydrates (mono-, di-, tri- or tetra-saccharide and hexose or pentose) or other substituents present, the stereochemical assignment of terminal monosaccharide units, the sequence of the glycan part, interglycosidic linkage and attachment points of the substituents to aglycones (Cuyckens, and Claeys, 2004). LC-MS methods based on electrospray ionisation are used for analysis of flavonoids (Laven, 2005).

LC-MS became efficient and popular after the development of atmospheric pressure ionisation (API) and is an easy technique for online analysis of flavonoids. There are three types of API sources: electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI). Among

these the most sensitive is ESI, but it is not universal. For some applications APCI is more suitable. ESI can operate in positive ion mode and negative ion mode. For flavonoids negative ion mode is most suitable because they are phenolics and phenolics readily ionise to give negative ions (Cuyckens and Claeys, 2004).

1.6 Antioxidant activity

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation and propagation of oxidising chain reactions (Velioglu *et al.*, 1998). There are two basic categories of antioxidants: natural and synthetic. Synthetic antioxidants in general, are compounds with phenolic structures having various degrees of alkyl substitution. Natural antioxidants on the other hand can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids and amines), or carotenoids (β-carotene, astaxanthin, lycopene, lutein) as well as ascorbic acid (Velioglu, *et al.*, 1998). Synthetic antioxidants like butylated hydroxylanisole (BHA) and butylated hydroxytoluene (BHT) have been used as antioxidants in foods (Williams *et al.*, 1999) but restrictions have been imposed on their use because of concern about their proven carcinogenicity in animal models, such as in rats (Ito *et al.*, 1983).

The importance of a high consumption of secondary plant products, which are widely distributed in fruits and vegetables, in reducing the incidence of degenerative diseases like cancer and arteriosclerosis has been demonstrated in several epidemiological studies (Schlesier *et al.*, 2002). Vegetarians and non-vegetarians have reduced blood pressure that correlates with a high intake of fruits and vegetables (Wang *et al.*, 1996).

Fruits and vegetables can provide protection against cancer and cardio- and cerbro-vascular diseases, attributed to the various antioxidants present (Wang *et al.*, 1996).

There is much evidence to indicate that free radicals cause oxidative damage to lipids, proteins and nucleic acids. Free radicals may be the principal cause of the natural history of a number of diseases (Wang *et al.*, 1996). Antioxidants, which can neutralize free radicals, may be of central importance in the prevention of these disease states. Increased risk of cancer mortality has been associated with low plasma levels of antioxidant vitamins (Wang *et al.*, 1996). Low plasma vitamin E and C levels correlate with increased mortality from ischaemic heart disease, according to cross-culture epidemiological studies (Wang *et al.*, 1996).

Fruits and vegetables contain many different antioxidant components and the majority of their antioxidant capacity may be from compounds other than vitamin C, vitamin E or β-carotene. Some of the frequently encountered components in the human diet, like flavonoids (including flavones, isoflavones, flavonones, anthocyanins, catechin, and isocatechin), demonstrate strong antioxidant activities (Wang *et al.*, 1996). Many natural antioxidants, especially flavonoids, exhibit a wide range of biological effects, including antibacterial, antiviral, antiinflammatory, antiallergic, antithrombotic and vasodialatory actions (Velioglu *et al.*, 1998). There is a consistency with the epidemiological studies which shows a significant positive association between intake of fruits and vegetables and reduced rate of heart disease mortality, common cancer and other degenerative diseases as well as ageing (Dillard and German, 2000). There is strong evidence for a reduced risk of cancer of the mouth, pharynx, oesophagus, lung, stomach, and colon. The data also gives strong support to a protective role for fruits and vegetables against pancreatic, bladder and breast cancers (Glade, 1997).

Many analytical methods have been developed to determine the antioxidant activity in all kinds of matrices like plasma, beverages, vegetables and fruits. These assays typically measure the ability of the test substances to reduce pro-oxidants. They use various radicals or metal ions as oxidants (Schlesier *et al.*, 2002). There are many different assays utilizing different approaches based on three common methods: β-carotene bleaching, DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) reduction; and the FRAP (ferric reducing and antioxidant power) method. The DPPH method utilises colorimetric changes following the direct reduction of DPPH radicals by antioxidants. The β-carotene bleaching method involves the inhibition of lipid peroxidation and subsequent bleaching of β-carotene, by antioxidants. The FRAP method acts by antioxidant-induced inhibition of the reduction of ferric to ferrous ion and complextion with FRAP reagent.

Vitamin C (ascorbic acid) is a water soluble antioxidant. It is oxidised easily to a free radical, semidehydroascorbic acid that is relatively stable. Further oxidation generates dehydroascorbic acid then diketogluconic acid, which has no biological function as an antioxidant (Kaur and Kapoor, 2001). Ascorbic acid shows antioxidant activity because of its ability to lose electrons, making it very effective in biological systems. The compounds in the water soluble portion of cells and tissues are protected and ascorbic acid reduces tocopherol radicals back to their active form at the cellular membranes (Kaur and Kapoor, 2001). Vitamin C deficiency and low intake of fruits and vegetables in people have been correlated with increase risk of oesophageal, pancreatic and lung cancer. It exacerbates atherogenesis in animal models (Wargovich, 2000).

The consumption of fruits and vegetables has been shown in epidemiological studies to reduce the risk of developing chronic diseases like cardiovascular diseases, cancer, diabetes and Alzheimer's disease. The phytochemicals, especially phenolics, in fruits and vegetables are thought to contribute to this reduced risk because of their antioxidant and antiproliferative (inhibition of cell growth) activities (Sun *et al.*, 2002). Phenolics exist in both soluble free and bound forms. Bound phenolics are mainly in the form of glycosides. They may survive the digestive processes of the stomach and small intestine and reach the small intestine intact and exhibit their bioactivity (Sun *et al.*, 2002).

Chapter 2: Chemical investigation of *Carpobrotus* species.

2.1 Objectives

The aim of these investigations was to define some of the chemical properties and to investigate some chemical constituents of the plant juices, methanolic extract, DCM extract and plant juice fractions of three *Carpobrotus* species: *C. edulis, C. rossii and C. aequilaterus.*

2.2 Reagents

Chemicals used in this investigation included sodium hydroxide and potassium hydrogen phthalate (Sigma-Aldrich, Sydney), sodium chloride (BDH, Poole, England), potassium chloride, hexane, acetic acid, ammonium hydroxide and sodium bicarbonate AR (analytical reagent) grade (Ajax, Sydney, Australia), phenolphthalein (By-Products and Chemicals Pty Ltd, Sydney, Australia), acetonitrile HPLC grade (J.T.Baker, USA), methanol LR (laboratory reagent) grade (BDH, Australia), sulphuric acid AR grade, dichloromethane (DCM) LR grade (Polytreat, Australia) and hydrochloric acid AR grade (BDH/Merck, Australia).

2.3 Equipment

Liquid chromatography - mass spectrometry (LC-MS) was performed using a Waters Alliance 2690 high performance liquid chromatograph (HPLC) and Waters 996 photodiodearray (PDA) detector coupled to a Finningan LCQ ion trap mass

spectrometer. The elemental analyser used was a Thermo Finningan Flash EA 1112 series. The pH meter used was by TPS Pty Ltd, Brisbane, model 1852 mv. The solid phase cartridges used were 100 mg/6 ml Strata-X cartridges from Phenomenex, Australia. The Muffle furnace used was from Carbolite, England. The flame photometer for sodium and potassium analysis was by Jenway Limited, Clinical Model PFP7, United Kingdom. The centrifuge used was from Clements Pty Ltd, Sydney, Australia. SPME fibres used were Supelco 75 μm Carboxen-PDMS , Supelco manual SPME syringe 85 μm Supelco Polyacrylate. GC-MS was performed using a Varian 3800 GC coupled to a Varian 1200 triple quadrupole MS using a Varian 'Factor Four' VF-5ms (30 m x 0.25 mm internal diameter and 0.25 μm film) GC column and Varian 1177 split/splitless injector in splitless mode. The analytical HPLC system consisted of a Prostar 210 solvent delivery system, Alltech 2000ES ELSD (evaporative light scattering detector) and Prostar 410 autosampler (Varian Inc, Melbourne, Australia) with a Waters Nova-Pak® C18 (150 mm x 3.9 mm). Alltech Prevail® Carobohydrate ES column (5 μm, 250 x 4.6mm; Alltech, Sydney, Australia).

2.4 Methods

2.4.1 Collection of plant material

Carpobrotus species plant material was collected from various parts of Tasmania. The plant material was collected from a single plant and then placed in plastic bags. The bags of plant material were carried at ambient temperature to the university and stored in a freeze at -18 $^{\circ}$C until extracted. Voucher specimens were lodged with the Tasmanian Herbarium, Hobart, Tasmania for preservation and identification. The

different species, their location of collection and the Tasmanian Herbarium voucher numbers for the collections are shown in Table 2.1.

Table 2.1 Different *Carpobrotus* species collected for use in the current study, location of their collection site, herbarium voucher number and codes for plant juices extracts.

Species	Location	Date	Voucher	Juice code
C. edulis	Site 1[a]	Oct-2004	HO 529460	CeJ1
	Site 2[b]	Oct-2004		CeJ2
	Site 3[c]	Oct-2004		CeJ3
C. rossii	Site 4[d]	Dec-2004	HO 529461, H0 529462	CrJ1, CrJ2, CrJ3
C. aequilaterus	Site 1	Nov-2005	HO 540318	CaJ1, CrJ2, CrJ3

[a]Victoria Esplanade, Bellerive. [b]street planting outside 23 Scott Street, Bellerive. [c]garden planting back yard, 23 Scott Street, Bellerive. [d]Seven Mile Beach, Tasmania.

2.4.2 Extraction of plant juice

Succulent leaves were removed from fresh plant material, cut into small pieces and macerated in a blender. The juice was decanted into a flask, and then centrifuged to remove suspended solid materials. The plant juices, thus prepared, were analysed for their chemical constituents. The methanolic and DCM extracts of the plant were prepared by soaking the small pieces of fresh plant material remaining after extraction of plant juice in methanol and DCM respectively for 24 hours and then decanting the solvents. Then extracts thus obtained were concentrated by rotary evaporation at reduced pressure.

2.4.3 pH

Plant juice that was obtained was pinkish in colour and was acidic in nature. The pH of the plant juice was tested using a pH meter.

2.4.4 Titration

A simple acid-base titration was performed to find out the acidity of the plant juice. Sodium hydroxide solution (0.1 M) was taken in a burette and, using phenolphthalein as indicator, plant juice was titrated in a conical flask. The endpoint was detected by colour change to a more intense pink. Sodium hydroxide solution was standardised using potassium hydrogen phthalate as primary standard.

2.4.5 Solid content by evaporation

The amount of dissolved solid present in the juice was analysed by taking a known volume of plant juice in a pre-weighed evaporating dish. This dish was left uncovered, in a fume cupboard. When most of the liquid was evaporated, it was kept in an oven at a temperature of 35–40 °C for removal of traces of liquid, and weighed. Drying was continued until it had a consistent weight.

2.4.6 Ash analysis

The amount of organic and inorganic matter present in the plant juice was determined by ash analysis. A know volume of the plant juice was taken and evaporated in a pre-weighed evaporating dish. It was dried to a solid mass and then it was burnt to ashes

in a muffle furnace at a temperature of 600–800 °C. At two hour time intervals, the evaporating dish was taken out cooled in a desiccator and weighed till there was no further loss of mass and only inorganic matter remained.

2.4.7 Elemental analysis

The plant juice was evaporated to a solid mass and then was subjected to elemental analysis. The percentage of carbon, oxygen, nitrogen and sulphur was determined.

2.4.8 Measurement of sodium and potassium ion concentrations in *Carpobrotus* species plant juice

The sodium and potassium ion content in the *carpobrotus* plant juice was determined using flame photometry.

2.4.8.1 Sodium determination

Dry analytical reagent quality NaCl (0.5846 g) was weighed and made up to 100.0 ml with distilled water to give a stock standard solution 100 mM in Na^+. The stock solution was diluted to 80, 60, 40 and 20 mM in Na^+. Standard solutions were analysed by flame photometry.

2.4.8.2 Potassium determination

Dry analytical reagent quality KCl (0.745 g) was weighed out accurately and made up to 100.0 ml with distilled water to give a stock standard solution of 100 mM in K^+.

The stock solution was diluted to 80, 60, 40 and 20 mM in K^+. Standard solutions were analysed bt flame photometry.

2.4.8.3 Preparation of samples of plant juice

Samples of plant juice were diluted five times with water and then passed through the flame photometer for sodium ion concentration determination. Undiluted plant juices were passed through the flame photometer for potassium ion concentration determination.

2.4.9 Analysis of flavonoids in *Carpobrotus* species using LC-MS

The methanolic extracts, DCM extracts and plant juices of *Carpobrotus* species were subjected to solid phase extraction before LC-MS analysis. For solid phase extraction 100 mg Phenomenex Strata-X cartridges® were used. Cartridge conditioning consisted of the addition and dilution of 1 ml of methanol followed by 1 ml of 5% methanol/water. Then 100 µl of sample was added to 1 ml of water and applied to the cartridge. The cartridge was washed with 1 ml of 5% methanol/water. The sample for analysis was eluted with 1 ml methanol and the eluent was collected a in glass vial for analysis by LC-MS. Elution was performed under positive pressure applied using a 10 ml plastic Luer-lock syringe.

LC-MS was used for the analysis of constituents in the methanolic extract, DCM extract and plant juice of *C. edulis*; methanolic extract and plant juice of *C. rossii*; and methanolic extract and plant juice of *C. aequilaterus*. The column used was a Waters Nova-Pak® C18 with flow rate 0.8 ml/min. Mobile phases used were water (A)

methanol with 2% acetic acid (B) and hexane (C). A linear gradient elution profile was followed from 80% A and 20% B to 100% B over 25 minutes; then to 80% B and 20% C over 1 min; then held at this composition for a further 4 minutes.

Mass spectra were acquired using negative ion electrospray ionization with 4KV needle voltage. Capillary temperature was 275 °C, sheath gas was nitrogen at 80 psi and auxillary gas was nitrogen at 40 psi. Ions in the range *m/z* 230 to 1500 were acquired, with altering data-dependent MS/MS product ion scan.

2.4.10 Analysis of flavonoids in *Carpobrotus* species by using HPLC.

High performance liquid chromatography with diode array detection (HPLC-DAD) was used for the analysis of chemical constituents in the crude plant juice and fractions of the plant juice obtained using solid phase extraction on a reverse phase coloumn. *C. rossii* plant juice was passed through a Phenomenex Strata-X cartridge® and sequentially eluted with 1 ml aliquots of 100% water, 20% methanol/water, 50% methanol/water, 80% methanol/water and 100% methanol. These fractions were analysed by HPLC to determine which fraction contained the flavonoids from the plant juice based on the characteristic ultra-violet (UV) spectra of flavonoids. The *Carpobrotus* species plant juices samples prepared for LC-MS analysis were also analysed by HPLC.

The column used was Waters Nova-Pak® C18 with flow rate 0.8 ml/min. The mobile phases used were water with 2% acetic acid (A) and methanol with 2% acetic acid (B). A linear gradient elution profile was followed from 80% A and 20% B to 100% B over 30 minutes.

2.4.10.1 Anti-microbial assay of flavonoids

Anti-microbial assays were performed in the laboratory of Munro and Blunt, Chemistry Department, University of Canterbury, New Zealand.

Culture of microbes: bacteria and fungi at a known concentration were mixed with Mueller Hinton or potato dextrose agar and were applied to Petri dishes so that after incubation a "lawn" of bacteria/fungi would grow over the dish.

Six organisms tested against were:

Bacteria - *Escherichia coli.* (Gram-negative ATCC 25922)

Bacillus subtilis. (Gram-positive ATCC 19659)

Pseudomonas aeruginosa. (Gram-negative ATCC 27853)

Fungi - *Candida albicans.* (ATCC 14053)

Trichophyton mentagrophtyes. (ATCC 28185)

Cladosporium resinae

Assay procedure: samples of *C. rossii* plant juice fractions prepared as described in 2.4.10 and *C. aequilaterus* plant juice and methanolics extract (33 mg/ml) were pipetted onto 6 mm diameter filter paper discs and their solvents were evaporated. The discs were then placed onto Mueller Hinton or potato dextrose seeded agar dishes (with appropriate solvent and positive controls) and incubated for 3-4 days.

If a sample showed any activity against the bacteria/fungi, then there was a zone of inhibition outside the disc. This was measured in millimetres as the radius of inhibition and was recorded for each bacteria/fungus. If there was no zone of inhibition then there was no anti-microbial activity for that bacterium/fungus.

2.4.11 Hydrolysis of *C. rossii* flavonoids and analysis by HPLC.

For the isolation of the flavonoid aglycone from *C. rossii,* the methanolic extract was subjected to acid hydrolysis. Acid hydrolysis was trialled using three different methods.

An aliquot (10 ml) of *C. rossii* methanolic extract solution containing 0.3 g of extract was transferred to a round bottom flask (250 ml) and 50 ml of 2 M HCL and 50 ml methanol were added. The mixture was refluxed for 2 hours and then the methanol was evaporated by vacuum rotary evaporation. (Leucuta *et al.*, 2005). In another method 10% glacial acetic acid was added instead of the methonolic HCl, and refluxed for 12-24 hours (Imperato, 1976). In another process 2M Sulphuric acid was used with 2 mg of ascorbic acid as an antioxidant. The mixture was heated at 80 °C for 2 hours (Sakai, 1987).

From the solutions obtained using each of the processes described above, 1 ml was taken and neutralized with sodium hydroxide solution. The samples were passed through a Phenomenex Strata-X cartridge® as described previously (in section 2.4.10) in order to obtain a flavonoid-rich fraction. The fractions collected were analysed by HPLC for flavonoid content.

2.4.12 Analysis of *Carpobrotus* species volatiles by using GC-MS with SPME

The SPME (Solid Phase Micro Extraction) technique was used for analysis of the volatile plant juice constituents of *Carpobrotus* species. Leaves of all the three plant species were taken and macerated in a clean glass mortar to obtain fresh juice. A sample of freshly collected plant juice was transfered into a glass vial and sampled by SPME using a Supelco 75 μm Carboxen-PDMS fibre. The three plant juices were left at room temperature for a week and again sampled for volatile constituents, this time using an

85 μm Supleco Polyacrylate fibre. SPME fibres were exposed to the headspace above the sample at room temperature in the sealed vial for five minutes. The fibres were desorbed and the volatiles analysed by GC-MS. Injector Temp was 280 °C with a desorption time of 2 minutes. The GC oven temperature progress was 35 °C for 2 minutes then to 280 °C at 10 °C per minute. Carrier flow rate was 1.2 ml per minute in constant flow mode with the split valve turned on at 2 minutes with MS scanned from *m/z* 35 to 350 every 0.3 seconds. Typical multiplier setting was 1000 V. Data was processed with Varian Star software.

2.4.13 Analysis of carbohydrates using HPLC with ELSD (Evaporative Light Scattering Detector)

Carpobrotus species juice samples for analysis of carbohydrates were diluted 1:20 with distilled water. Aliquots of 100 μl of internal standard (sorbitol 5 mg/ml in water) were then added to 1 ml aliquots (or equivalent ratios for smaller volumes) of diluted juice extract. A carbohydrate standard solution containing sucrose, glucose and fructose at 15 mg/ml was also analysed.

ELSD conditions were tube temperature 93 °C, nitrogen flow 2.4 l/min, gain of 1 with impactor off. Fixed loop injection (10 μl) was used. The mobile phases consisted of acetonitrile (A) and 0.04% ammonium hydroxide in water (B) with linear gradient from 17% B at 0 minutes, to 27% at 25 minutes, to 45% at 40 minutes, and then to 55% at 60 minutes.

2.5 Results and discussion

2.5.1 Extraction of plant juice

All the three plant juices were extracted. *C. edulis* yielded 80% v/w, *C. rossii* yielded 79% v/w and *C. aequilaterus* yielded 80% v/w of plant juice.

2.5.2 Chemical analyses

Chemical properties including acid content, solids on evaporation, ash analysis, elemental analysis and pH of the three *Carpobrotus* species plant juices are summarized in Table 2.2.

Table 2.2 Chemical properties of three *Carpobrotus* species plant juices

Parameters	*C. edulis*	*C. rossii*	*C.aequilaterus*
pH	4.7	4.73	4.66
Acid content	0.0038 N	0.0070 N	0.0076 N
Solids on evaporation	0.0260 g/ml	0.0335 g/ml	0.0307 g/ml
Ash analysis	0.072%w/v	0.557%w/v	0.659%w/v
Elemental analysis			
Carbon	23.35%	11.28%	13.19%
Hydrogen	3.78%	3.20%	2.46%
Nitrogen	0	0.28%	0.51%
Sulphur	0	0	0

2.5.3 Sodium and potassium levels

The calibration curve of the sodium ion analysis is shown in Figure 2.1 and the concentration of sodium ions in *Carpobrotus* species is shown in Table 2.3.

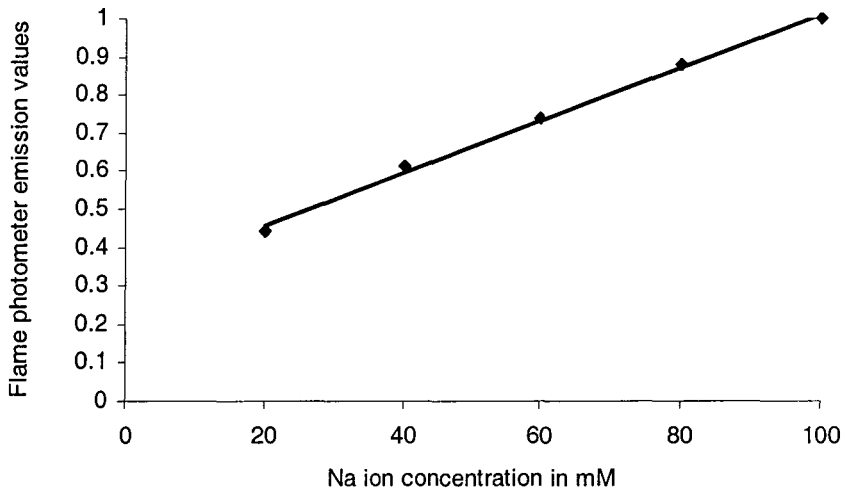


Figure 2.1 Calibration curve of Na^+ measured by flame photometry ($r^2 = 0.996$).

The calibration curve of the potassium ion is shown in Figure 2.2 and the concentration of the potassium ions in *Carpobrotus* species is shown in Table 2.3.

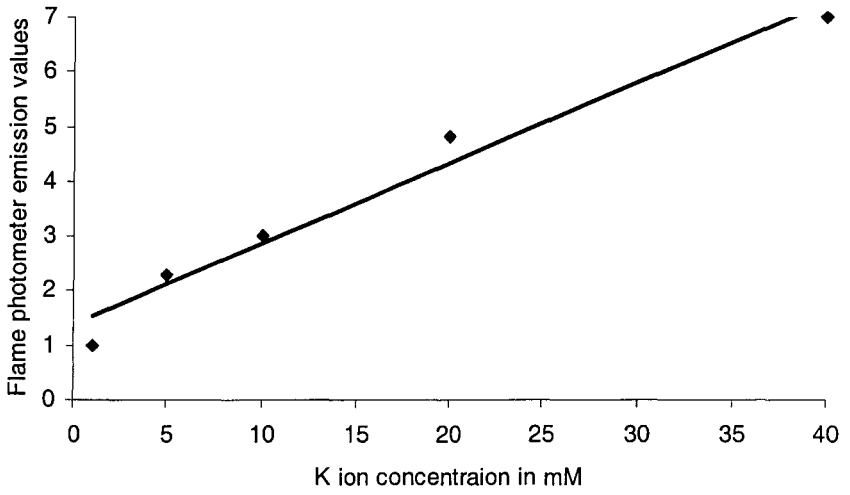


Figure 2.2 Calibration curve of K^+ measured by flame photometry ($r^2 = 0.971$).

Table 2.3 Sodium and Potassium ion concentrations in the juices from different *Carpobrotus* species.

Carpobrotus plant codes	Diluted sample photometric emission [Na^+]	[Na^+] mM	Juice [Na^+] mM	Sample photometric emission	Juice [K^+] mM
Ce[a]J1	0.56	34	174	2.49	7.53
CeJ2	0.41	13	66	2.93	10.52
CeJ3	0.36	6	30	3.14	11.94
Cr[b]J1	0.69	53	266	3.85	16.76
CrJ2	0.66	49	245	1.67	1.97
CrJ3	0.62	43	216	1.49	0.75
Ca[c]J1	0.66	49	245	2.72	9.09
CaJ2	0.66	49	245	3.70	15.74
CaJ3	0.67	50	252	2.38	6.79

[a] *C. edulis*, [b] *C. rossii* and [c] *C. aequilaterus*.

The *C. rossii* and *C. aequilaterus* plant juices had no inter- or intra-species variation in the concentration of sodium. *C. edulis* was collected from different sites, including a coastal area, a street planting and a home garden, while the *C. rossii* and *C. aequilaterus* juices came from different plants from the same sites. *C. edulis* collected from the garden site had a very low levels of sodium (6 mM) compared with that collected from the coastal sites (34 mM). *C. rossii* and *C. aequilaterus* juices had higher concentrations of sodium. The low levels of sodium found in *C. edulis* juice may be dependent on the salt level and other mineral levels of the soil in which they were grown.

The concentration of potassium varied considerably between plants, but there was a higher concentration of potassium in *C. edulis* juice CeJ3 when compared to its sodium level. This higher concentration may be attributed to the soil chemistry.

Cryophytum crystalinum, also called ice plant, is a well known salt tolerant plant. It is reported to accumulate high levels of salt (400-500 mM Na^+) (Denmig and Winter, 1986). *Carpobrotus* species salt levels were high (up to 266 mM Na^+) and it is possible that *carpobrotus* species may similarly be useful plants in saline environments.

2.5.4 Analysis of flavonoids in *Carpobrotus* species using LC-MS

The LC-MS chromatograms of *Carpobrotus edulis* methanolic extract (Figure 2.3), DCM extract and plant juice were quite similar. Chromatograms gave similar peaks at identical elution times. The MS data of the compounds present in the plant juice and methanolic extract were also very similar. The plant juice and methanolic extract of *Carpobrotus edulis* showed the following major peaks presented in Table 2.4.

Table 2.4 The retention time, molecular weight and UV data of the main peaks of *C. edulis* methanolic extract, by LC-MS.

Retention time (min)	Molecular weight	UV absorption maxima
8.06	772	259, 266(shoulder), 352.
9.42	640	259, 265(shoulder), 354.
10.43	948	255, 274, 298(shoulder), 337
10.93	654	257, 265(shoulder), 352

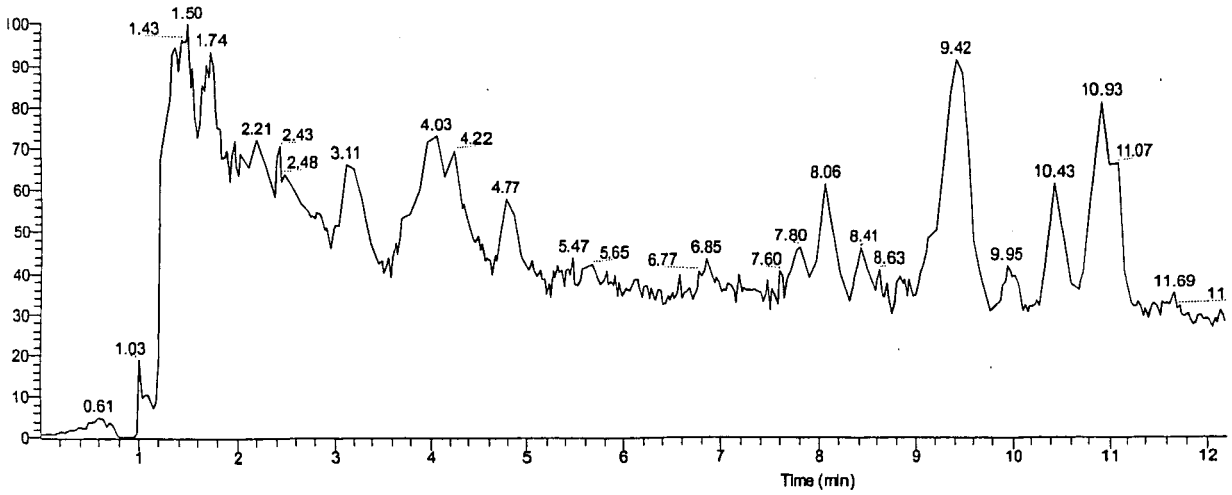


Figure 2.3 LC-MS chromatogram of *C. edulis* methanolic extract with detection by MS, showing the major flavonoid peaks that eluted between 8 and 11 minutes.

The LC-MS data from *Carpobrotus rossii* methanolic extract (Figure 2.4) and plant juice were quite similar. The plant juice and methanolic extract data from *C. rossii* showed the following major peaks presented in Table 2.5.

Table 2.5 The retention time, molecular weight and UV data of the main flavonoid peaks of *C. rossii* plant juice and methanolic extract by LC-MS.

Retention time (min)	Molecular weight	UV absorption maxima
9.49	640	
10.01	784	
10.48	784	257, 271(shoulder), 352
11.09	652	257, 271(shoulder), 352
11.40	960	233, 296(shoulder), 330

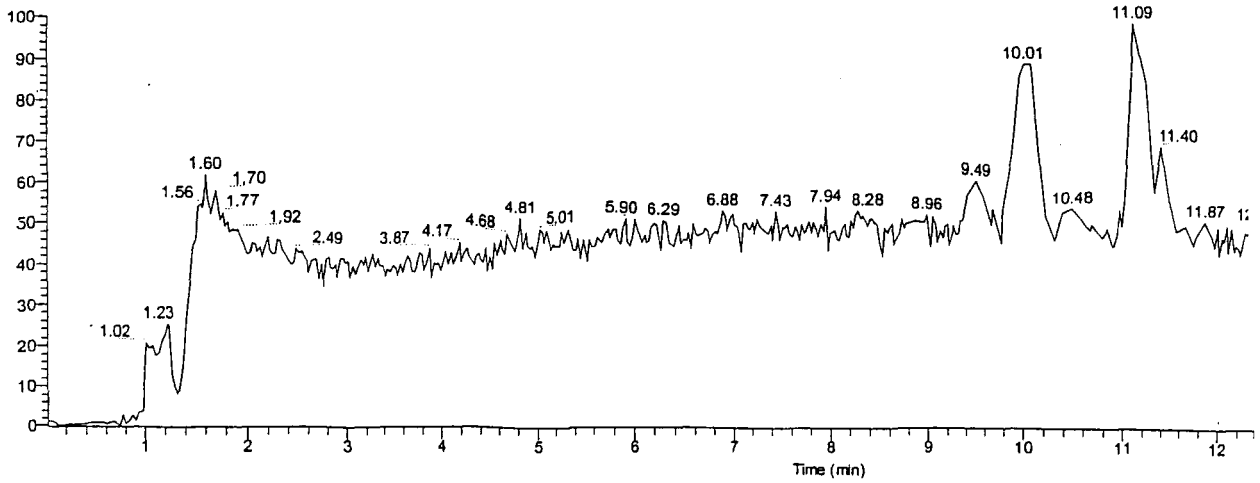


Figure 2.4 LC-MS chromatogram of *C. rossii* methanolic extract with detection by MS, showing the major flavonoid peaks.

The LC-MS data of *Carpobrotus aequilaterus* methanolic extract and plant juice were quite similar. The plant juice and methanolic extract data of *C. aequilaterus* showed the following major peaks presented in Table 2.6. Figure 2.5 shows the UV absorbance spectrum of the *C. aequilaterus* peak with retention time 11.23 minutes, which is a typical flavonoid spectrum with absorbance maxima at 257 nm and 354 nm. The LC-MS chromatogram of the methanolic extract is shown in Figure 2.6. This figure shows the chromatograms obtained by MS and by UV absorbance at 280 nm and 370 nm.

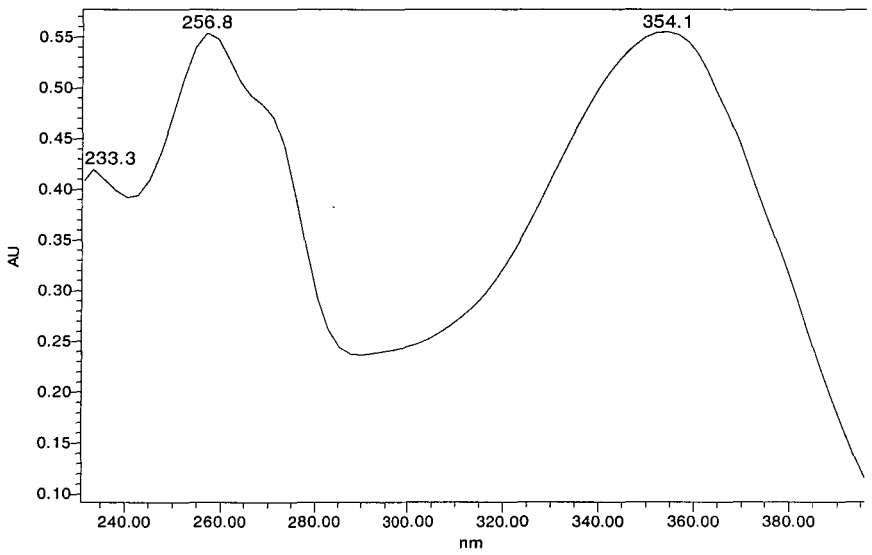


Figure 2.5 UV spectrum of *C. aequilaterus* peak with retention time 11.23 minutes.

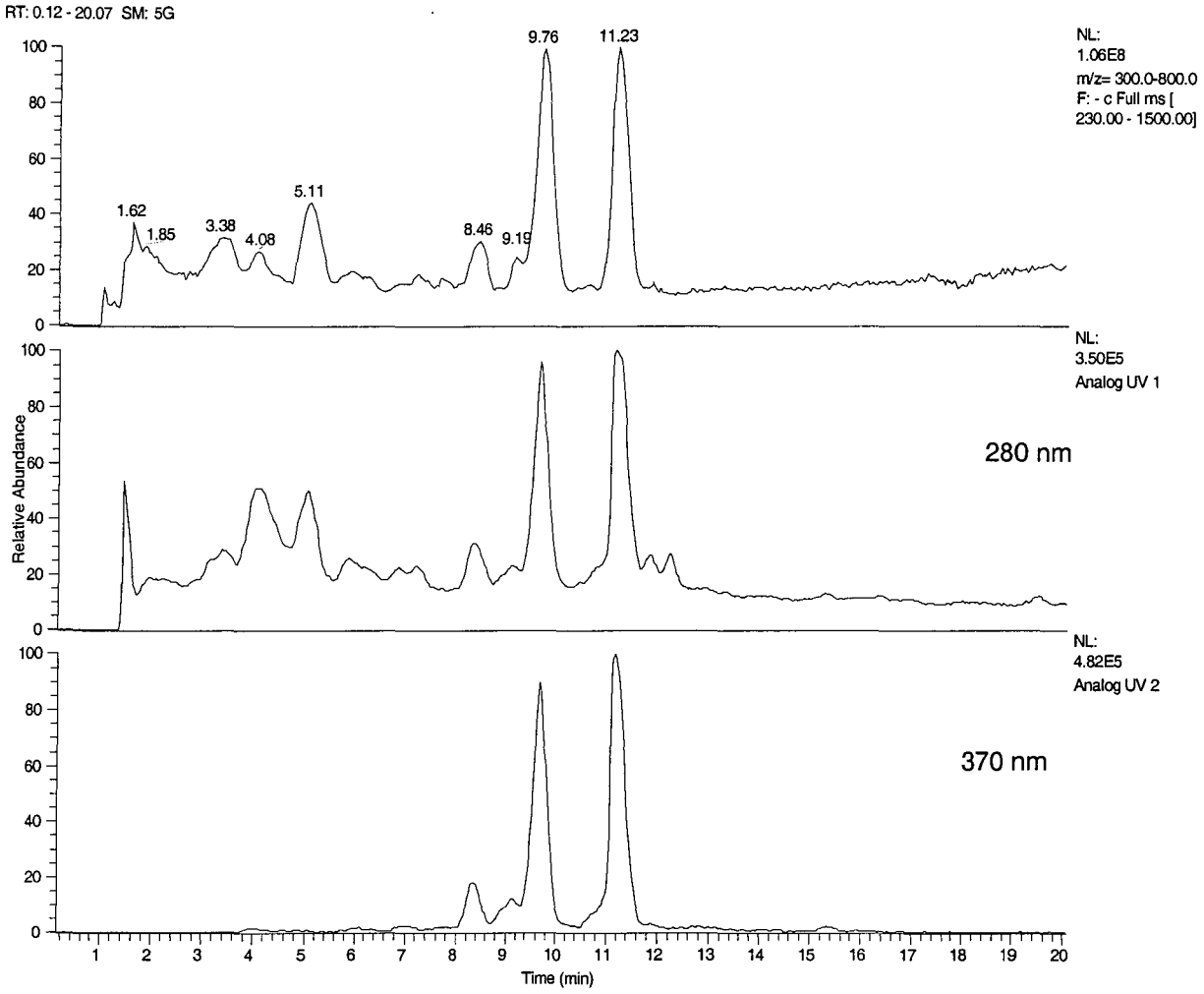


Figure 2.6 LC-MS chromatography of *C. aequilaterus* methanolic extract with detection by MS (upper chromatogram), at 280 nm (middle chromatogram) and at 370 nm (lower chromatogram) showing the major flavoniod peaks.

Table 2.6 The retention time, molecular weight and UV data of the main peaks of *C. aequilaterus* methanolic extract, by LC-MS.

Retention time (Min)	Molecular weight	UV absorption maxima
8.39	772	259, 349.
9.59	786	257, 352
9.76	640	257, 352
11.23	654	257, 354

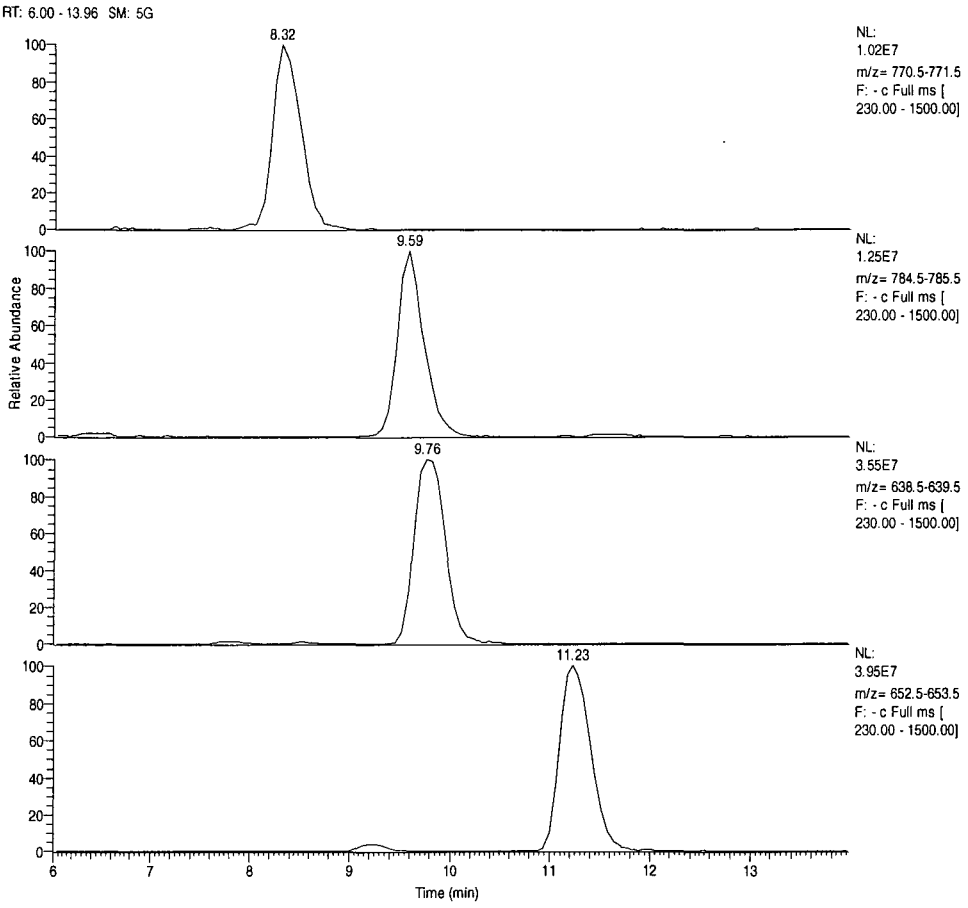


Figure 2.7 LC-MS chromatography of *C. aequilaterus* methanolic extract showing the flavonoid peaks and retention time. These peaks were obtained by m/z values of 771, 785, 639 and 653 peaks of chromatogram (Figure 2.5).

The compounds that eluted in the earlier part (retention time 0-6 minutes) of the chromatogram in all three *Carpobrotus* species are very polar and might be carbohydrates. The mass data indicated that these compounds had high molecular weights (1400 –1500), consistent with polysaccharides containing several sugar units.

Figure 2.7 shows that there are 4 major flavonoid peaks in the *C. aequilaterus* methanolic extract, whereas in Figure 2.6 there are only 3 flavonoid peaks evident. This is because the peak with retention time 9.76 min is a mixture of two flavonoids, one with retention time 9.59 min and another 9.76 min. These two peaks were not resolved chromatographically but could be resolved by observing selected masses (*m/z* 785 and *m/z* 639).

The molecular weights of the flavonoids and fragments obtained from each flavonoid, including data from secondary fragmentation after ion trapping (MS-MS or MS^2) detected in the plant juice and methanolic extract of *C. edulis* are given in Table 2.7.

Table 2.7 Data from MS of flavonoids from *C. edulis*, including molecular weight, major fragment mass and corresponding mass losses.

Molecular weight	fragments observed in MS^2 data	loss in mass
772	640, 332	132, 308
640	332	308
948	772,332	176, 440(132+308)
654	346	308

From the literature the molecular weights of different sugars are known. The molecular weight of glucose and galactose is 180; that of arabinose and xylose is 150 and rhamnose is 164. When these sugar units are bonded to a flavonoid, one water molecule is lost. So the mass gain when these sugars are bonded to a flavonoid is 162 for glucose and galactose, 132 for arabinose and xylose and 146 for rhamnose. Conversly, in MS, fragmentation involving loss of sugar groups from flavonoid glycosides results in equivalent mass losses. So MS can indicate, from the loss of mass from the flavonoid glycosides, which sugar may have been present. The data for *C. edulis* flavonoid glycoside are given in Table 2.8.

Table 2.8 Mass loss from *C. edulis* flavonoid glycosides, observed during mass spectrometry and possible carbohydrates responsible.

Mass loss	Possible carbohydrate
132	arabinose or xylose ($C_5 H_{10} O_5$)
308	rhamnose + glucose or galactose, rutinose. ($C_6 H_{12} O_5 + C_6 H_{12} O_6$)
176	glucuronic acid ($C_6 H_{10} O_7$)
440 (132+308)	arabinose or xylose + rutinose ($C_5 H_{10} O_5 + C_6 H_{12} O_6$, $C_6 H_{12} O_6$)

From the MS^2 data and loss in masses (Table 2.7 and 2.8) we can conclude the presence of two main flavonoid aglycone moieties having molecular weights (MW) 332 and 346. The UV data of these two flavonoids are consistent with their classification as a flavonol or flavonone. Three of the flavonoids possess the aglycone having MW 332 and one flavonoid possesses the other aglycone, having MW 346.

The flavonoid aglycone with MW 332 appears to be substituted with a disaccharide, possibly rutinose, to give the compound with molecular weight 640. An additional substitution of arabinose or xylose would give the compound with MW 772, and further substitution with glucuronic acid would give the compound with MW 948. There appears to be only one compound with the second flavonoid aglycone backbone (MW 346), which is consistent with a compound substituted with rutinose, or an equivalent disaccharide, to give the compound with MW 654.

The molecular weights of the flavonoids detected and fragments in the plant juice and methanolic extract of *C. rossii* are given in Table 2.9.

Table 2.9 Data from MS of flavonoids from *C. rossii*, including molecular weight, major fragment mass and corresponding mass losses.

Molecular weight	fragments observed in MS^2 data	loss in mass
640	346	294 (132+ 162)
784	640,346	144, 294 (132+162)
652	508	144
960	816, 346 (weak)	144, 614 (470)

The loss of mass from the flavonoid glycosides can be interpreted using similar arguments as for *C. edulis* flavonoids. There appears to be only one flavonoid aglycone with MW 346. The compound with MW 640 loses mass of 294 to give the aglycone. The loss of 294 corresponds to loss of 132 and 162, which is consistent with xylose or arabinose (132) and glucose or galactose (162). The compounds with MW 784, 652 and 960 lose 144 and this is not consistent with the loss of a sugar substituent. One possibility may be the loss of the 3-hydroxy-3-methylglutaric acid moiety. This acid has a molecular weight of 162 giving a mass difference of 144 when it acylates a flavonoid. Acylated flavonoids are known from the literature, but the 3-hydroxy-3-methylglutaric acid moiety is rare. The compound with MW 784 loses 144 and a further 294 to give the flavonoid aglycone. This compound is consistent with an addition of 144 to the compound with MW 640. The compound with MW 652 loses 144 to give a fragment of mass 508 which presumably consists of the aglycone and a sugar moiety with mass of 162 (glucose or galactose). The compound with MW 960 loses 144 and 470. The loss of 470 corresponds to loss of 2 lots of 162 (glucose or galactose) and 146 (rhamnose).

The molecular weight of the flavonoids and fragments detected in plant juice and methanolic extract of *C. aequilaterus* are given in Table 2.10.

Table 2.10 Data from MS of flavonoids from *C. aequilaterus*, including molecular weight, major fragment mass and corresponding mass losses.

Molecular weight	fragments observed in MS^2 data	loss in mass
772	640,332	132, 308
786	640,332	146,308
640	332	308
654	346	308

The loss of mass from the flavonoid glycosides can be interpreted using similar arguments as for *C. edulis* flavonoids. The flavonoids with MW 640, 654 and 772 have the same retention times (within experimental error) and the same fragmentation patterns by MS as the compounds from *C. edulis*. They appear to be identical to the compounds from *C. edulis*. The flavonoid with MW 786 is present only in *C. aequilaterus* and it appears due to the additional substitution of rhamnose to the compound with molecular weight 640.

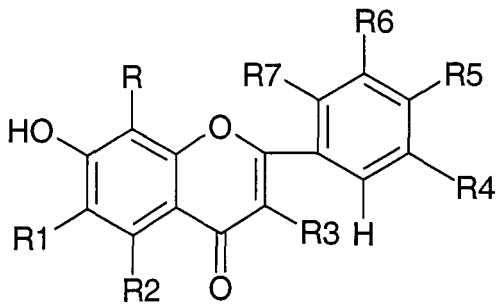


Figure 2.8 Basic structural skeleton of the flavonoid aglycone having molecular weight of 332.

Table 2.11 List of described or possible structures of flavonoids having molecular weight 332.

structures	R	R1	R2	R3	R4	R5	R6	R7	Reference
1	OH	OH	OH	OCH_3	H	OH	H	H	Ponce *et al.*, (2004)
2	H	H	OH	OH	OH	OCH_3	H	OH	Vvedenskaya *et al.*,(2004)
3	H	H	OH	OH	OCH_3	OH	OH	H	Rosler *et al.*, (2003)
4	OH	OH	H	OCH_3	OH	OH	H	H	Klopman *et al.*, (1988)
5	H	OH	OH	OH	OH	OCH_3	H	H	Greenham *et al.*, (2003)
6	H	OH	OH	H	OCH_3	OH	OH	H	Horie *et al.*, (1992)
7	OH	OH	OH	H	H	OH	OCH_3	H	Kurkin *et al.*, (1997)
8	H	H	OH	OH	H	OCH_3	OH	OH	Iinuma *et al.*, (1985)

The known potential structures of flavonoid aglycones having molecular weight 332, includes the following in Table 2.11. Figure 2.8 shows the basic structural skeleton of such flavonoids. In all the above potential structures it is possible for the sugar moieties to be attached at any hydroxyl group. One of the classic examples for the attachment of sugar moieties is rutin (Figure 2.9) which is a flavonol containing rutinose, a disaccharide moiety of molecular weight 308. It is known from mass data that some flavonoids present in *C. edulis* and *C. aequilaterus* contains a sugar moiety with a mass of 308, consistent with rutinose. However, rutin was not detected in any *Carpobrotus* species extract.

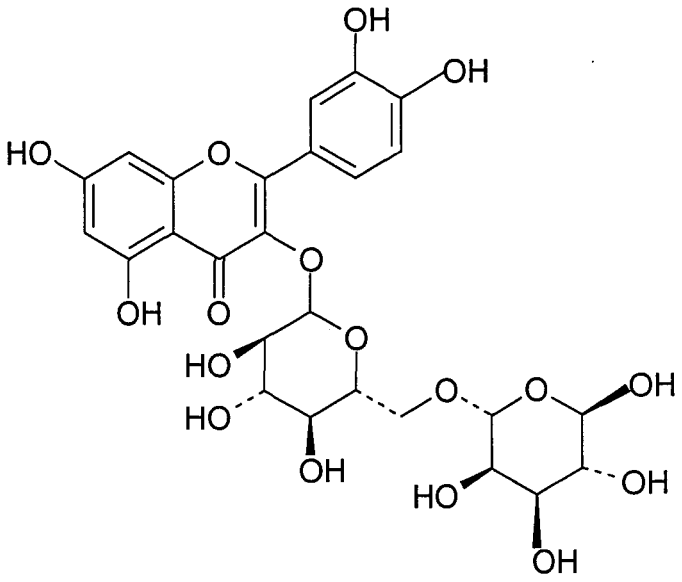


Figure 2.9 Structure of rutin having MW 610.

From the mass data, the flavonoid present in *C. edulis* and *C. aequilaterus* with molecular weight 772 has 3 sugar moieties. It can be assumed that the flavonoid may have a trisaccharide, or a disaccharide and monosaccharide, as sugar moieties. Fragmentation data indicating fragments corresponding to discrete mass losses of 132 and 308 suggest that it has a disaccharide and monosaccharide as sugar moieties. To determine the structure of the aglycone with MW 332 for *C. edulis* and *C. aequilaterus*, controlled hydrolysis, mass spectrometry, and NMR spectroscopy could be carried out. This was beyond the scope of the current study.

The known potential structures of flavonoids having molecular weight 346, includes the following in Table 2.12. Figure 2.10 shows the basic structural skeleton of such flavonoids.

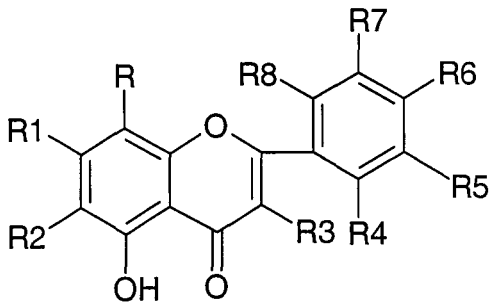


Figure 2.10 Basic structural skeleton of flavonoids having molecular weight 346.

Table 2.12 List of described or published structures of flavonoids having molecular weight of 346.

structures	R	R1	R2	R3	R4	R5	R6	R7	R8	Reference
1	H	OH	OCH_3	OCH_3	H	OH	OH	H	H	Triana *et al.*, (2003)
2	OCH_3	OH	H	H	OH	H	H	OH	OCH_3	Wang *et al.*,(2002)
3	H	OH	H	OH	H	OCH_3	OH	OCH_3	H	Guo *et al.*, (1998)
4	H	OH	OCH_3	OH	H	OCH_3	OH	H	H	Justesen *et al.*,(2001)
5	H	OCH3	H	OCH_3	H	OH	OH	OH	H	Matsuda *et al.*, (2002)
6	OCH_3	OH	H	OH	H	OCH_3	OH	H	H	Manthey *et al.*, (2004)
7	OH	OCH_3	OCH_3	H	H	OH	OH	H	H	Jamzad *et al.*, (2003)

In all the above potential structures it is possible for the sugar moieties to be attached at any hydroxyl group.

From the LC-MS data of extracts of *C. rossii* it was found that most of the flavonoids in it lose a fragment of mass 144, which is not so common. It has been found from the literature that one possibility is the 3-hydroxy-3-methylglutaric acid moiety. An example of its attachment to a flavonoid is presented in Figure 2.11. This compound was one of eight containing the 3-hydroxy-3-methylglutaric acid moiety isolated from two species of *Frullania.* (Kraut *et al.*, 1993).

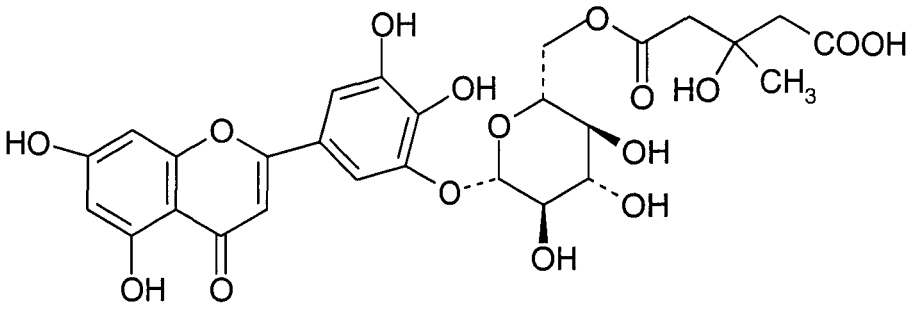


Figure 2.11 Structure of a flavonoid from *Frullania muscicola* containing the 3-hydroxy-3-methylglutaric acid moiety.

In summary:

- *C. edulis* and *C. aequilaterus* contained 4 flavonoids, with an aglycone having MW 332 (n=3) and 346 (n=1).
- None of the chemical constituents described from the South African *C. edulis* (van der Watt and Pretorious, 2001) were detected in the Tasmanian *C. edulis* samples.
- *C. rossii* contained 4 flavonoids with an MW 346.
- *C. rossii* it was found that 3 of flavonoids lose a fragment of mass 144, which is consitent with 3-hydroxy-3-methylglutaric acid moiety. All the flavonoids detected in *C. rossii* are novel.
- None of these 3 flavonoids were previously been identified.

2.5.5 Analysis of flavonoids in *Carpobrotus* species using HPLC

The nine plant juices, three from each species of *Carpobrotus* (*edulis, rossii* and *aequilaterus*) were analysed by HPLC for their flavonoid content, with UV detection.

The HPLC chromatograms of *C. edulis* plant species with UV spectrum maxima at 259 nm and 352 nm are shown in Figures 2.12, 2.13 and 2.14 with detection at 340 nm. Similarly the HPLC chromatograms for *C. rossii* are presented in Figures 2.15, 2.16 and 2.17, and *C. aequilaterus* in Figures 2.18, 2.19 and 2.20.

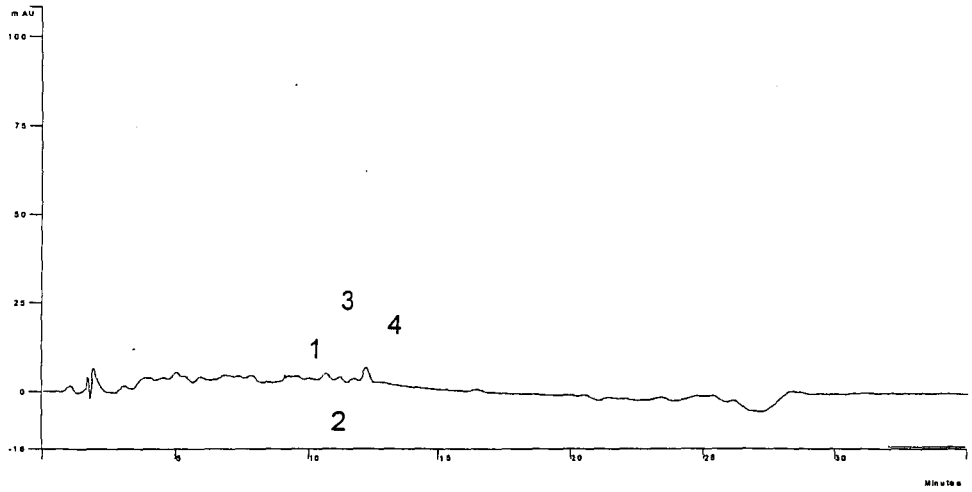


Figure 2.12. HPLC chromatogram with UV detection at 340 nm of *C. edulis* (CeJ1) plant juice.

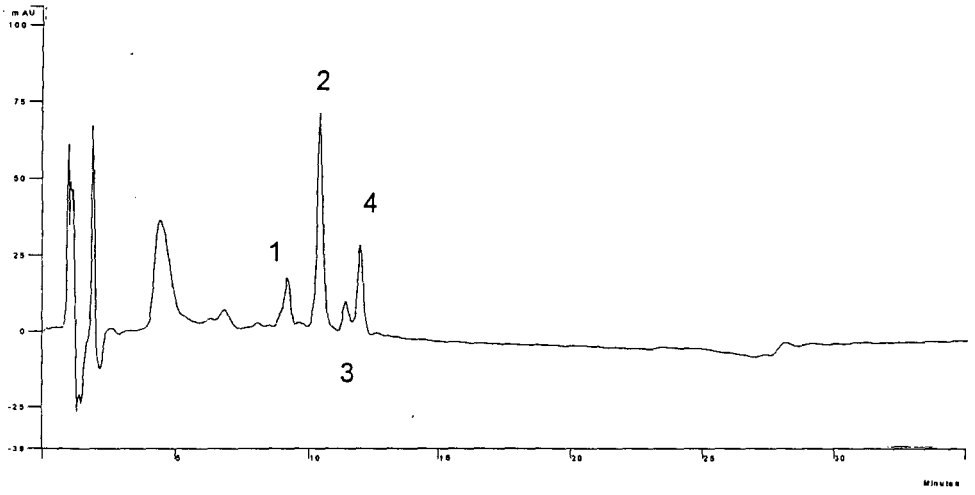


Figure 2.13 HPLC chromatogram with UV detection at 340 nm of *C. edulis* (CeJ2) plant juice.

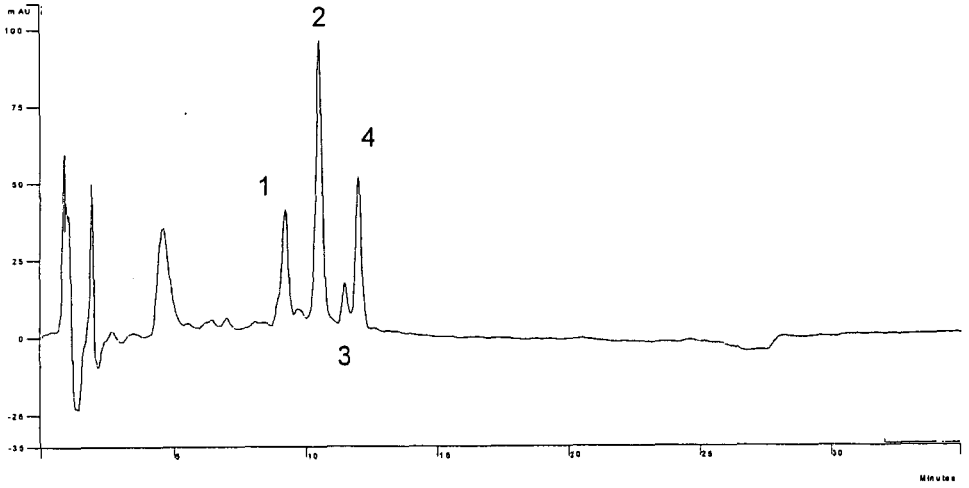


Figure 2.14 HPLC chromatogram with UV detection at 340 nm of *C. edulis* (CeJ3) plant juice.

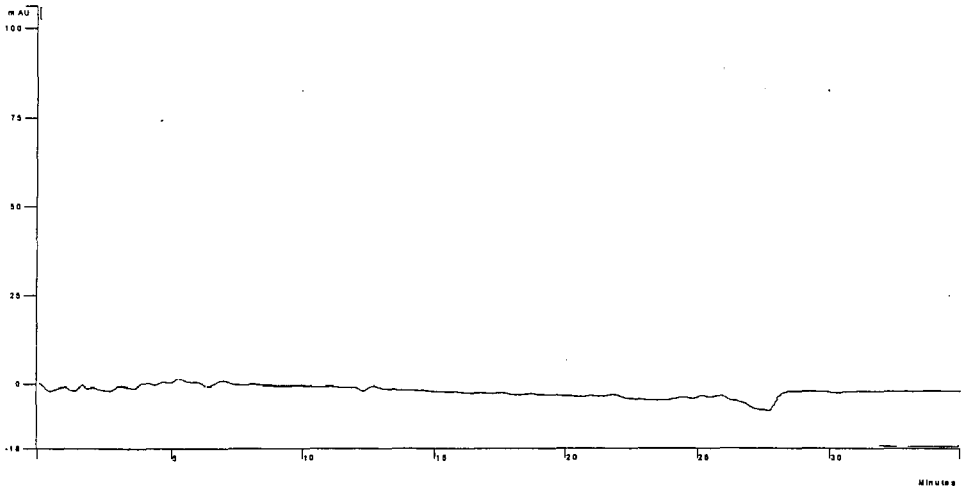


Figure 2.15 HPLC chromatogram with UV detection at 340 nm of *C. rossii* (CrJ1) plant juice.

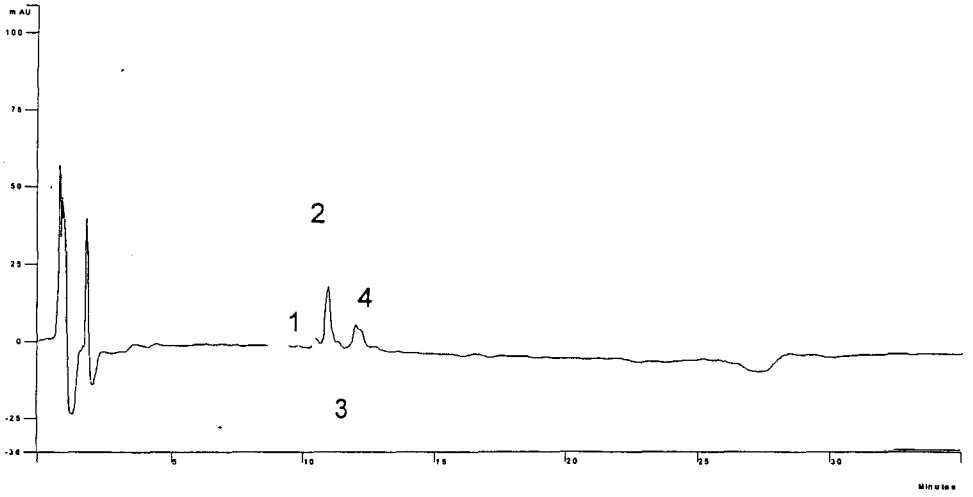


Figure 2.16 HPLC chromatogram with UV detection at 340 nm of *C. rossii* (CrJ2) plant juice.

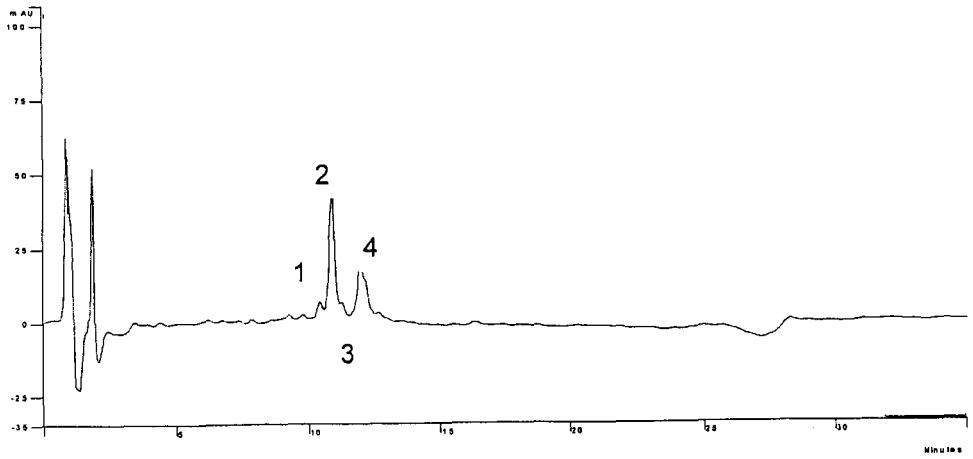


Figure 2.17 HPLC chromatogram with UV detection at 340 nm of *C. rossii* (CrJ3) plant juice.

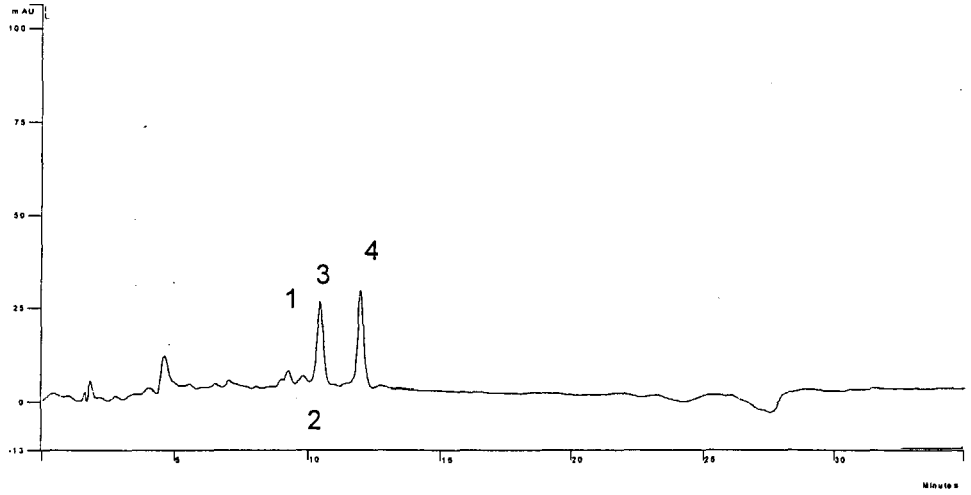


Figure 2.18 HPLC chromatogram with UV detection at 340 nm of *C. aequilaterus* (CaJ1) plant juice.

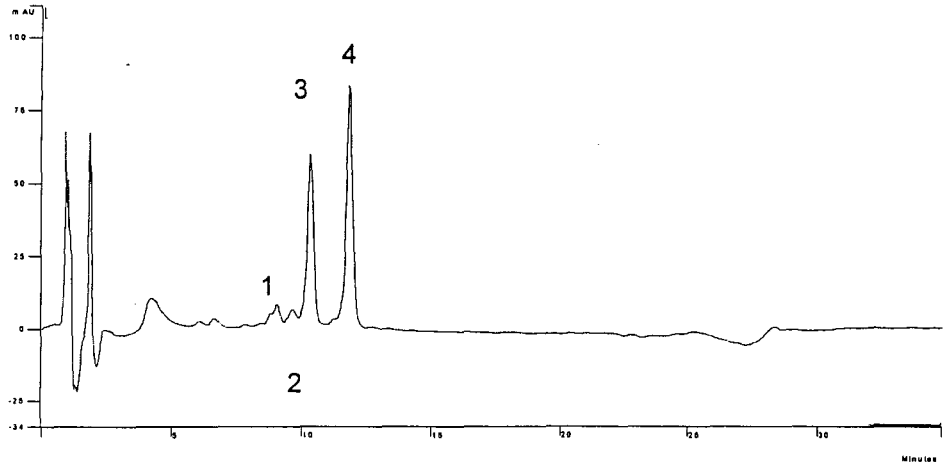


Figure 2.19 HPLC chromatogram with UV detection at 340 nm of *C. aequilaterus* (CaJ2) plant juice.

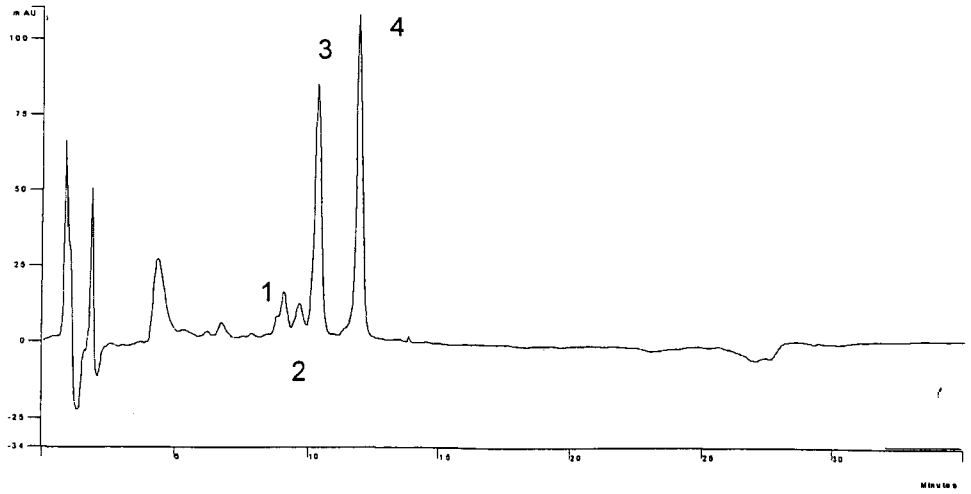


Figure 2.20 HPLC chromatogram with UV detection at 340 nm of *C. aequilaterus* (CaJ3) plant juice.

The HPLC chromatograms of the nine plant juices of *Carpobrotus* species show 4 major peaks corresponding to the flavonoids. The UV data support the presence of flavonoids, based on their UV profile from LC-MS (Figure 2.5) and their agreement with the peaks observed by LC-MS (Figures 2.3, 2.4, 2.6 and 2.7). From the above chromatograms there appears to be little within-species inter-plant variability in flavonoid profile. The HPLC chromatograms validate the identification of each plant since each species' flavonoid profile is consistent with its identification. To explore flavonoid variability within and between species, a survey of *Carpobrotus* species from different sites could be of value. The variability in flavonoids among the introduced and native species could be explored if plants were collected from more sites.

The HPLC chromatograms of *C. rossii* plant juice fractions are shown in Figures 2.21, 2.22, 2.23, 2.24 and 2.25. In the chromatograms the peaks that eluted at retention times between 10 and 15 minutes were flavonoids, based on UV and retention time data.

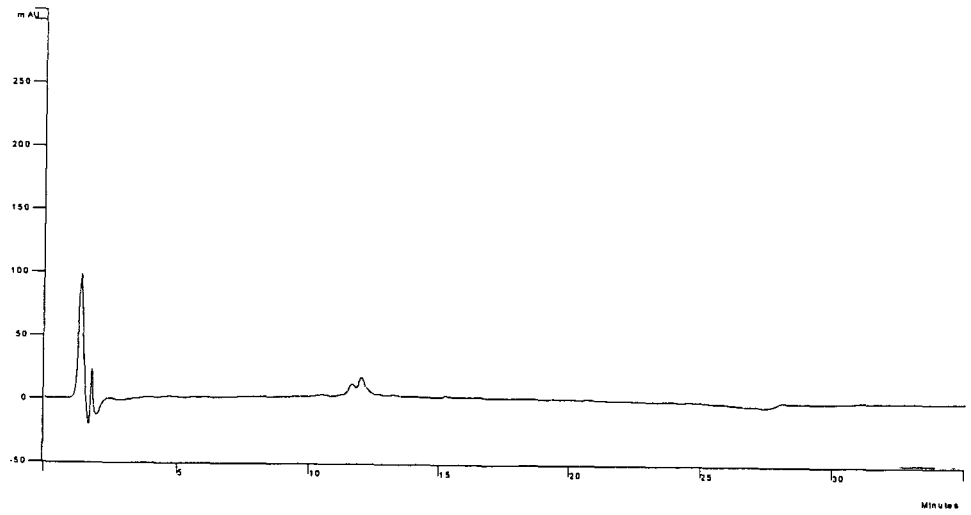


Figure 2.21 HPLC chromatogram with UV detection at 340 nm of 100% methanol solid phase elution fraction of *C. rossii* plant juice.

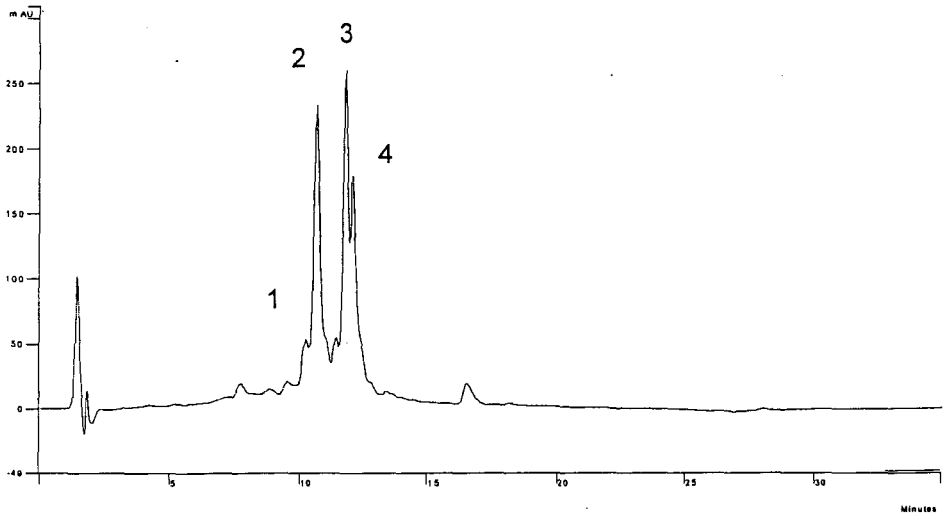


Figure 2.22 HPLC chromatogram with UV detection at 340 nm of 80% methanol solid phase elution fraction of *C. rossii* plant juice.

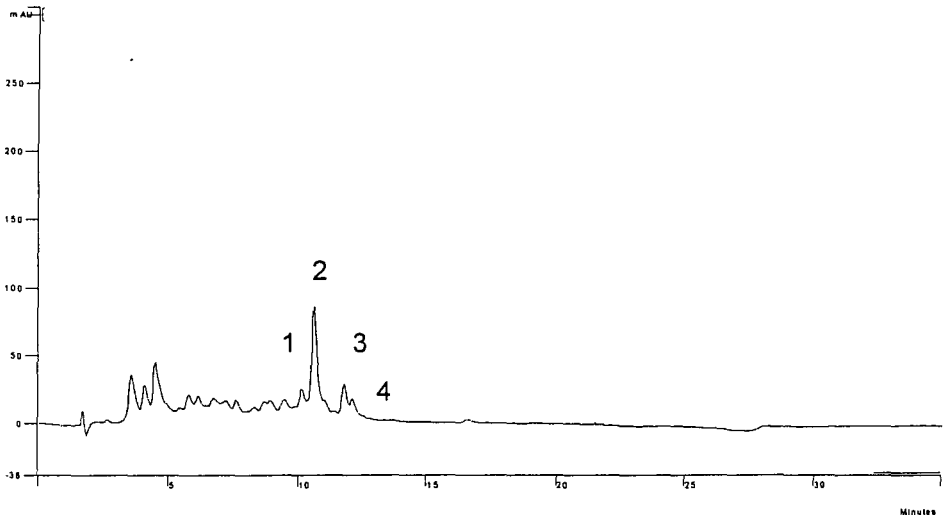


Figure 2.23 HPLC chromatogram with UV detection at 340 nm of 50% methanol solid phase elution fraction of *C. rossii* plant juice.

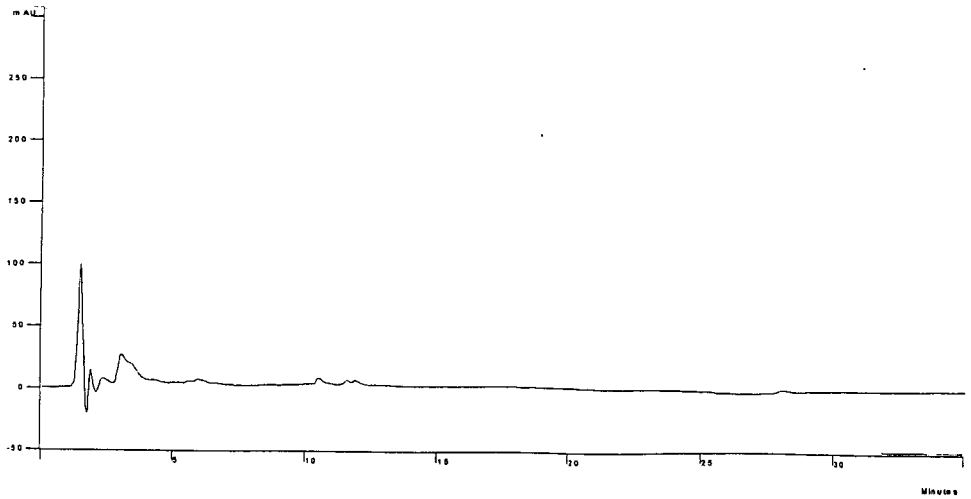


Figure 2.24 HPLC chromatogram with UV detection at 340 nm of 20% methanol solid phase elution fraction of *C. rossii* plant juice.

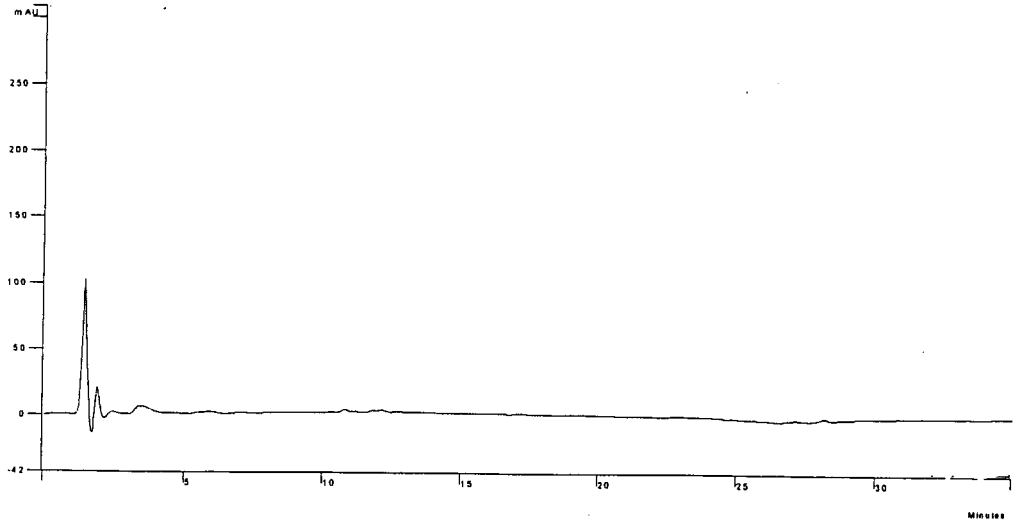


Figure 2.25 HPLC chromatogram with UV detection at 340 nm of 100% water elution from solid phase extraction of *C. rossii* plant juice.

From the chromatograms it was seen that the 80% methanol fraction from solid phase extraction of *C. rossii* plant juice had the highest concentration of flavonoids, followed by the 50% methanol fraction. Other fractions had no or very low amounts of flavonoids. This result indicates that for isolation of the flavonoids from the plant juice for further investigations, including identification, the 80% methanol fraction is the appropriate fraction to collect.

2.5.5.1 Anti-microbial assay of flavonoids

The anti-microbial assay of flavonoids present in the *C. rossii* plant juice fractions showed that the 100% methanolic fraction (zone of inhibition 1 mm) and 20% methanolic fraction (zone of inhibition 2mm) were active against *Trichophyton mentagrophtyes. C. aequilaterus* plant juice (zone of inhibition 2 mm) and methanolic extract (zone of inhibition 3 mm) were active against *Bacillus subtilis.* The antifungal activity of 20% and 100% methanolic fractions of *C. rossii* suggest that anti-microbial activity may not entirely be due to flavonoids but may be due to tannins and other metabolites. Studies on *C. edulis* showed the tannin fraction had higher anti-microbial activity than known flavonoids (van der Watt and Pretorious, 2001)

2.5.6 Analysis of flavonoids after hydrolysis of *C. rossii* methanolic extract, using HPLC

The HPLC chromatogram of *C. rossii* methanolic extract is shown in Figure 2.26. The chromatogram of a sample of *C. rossii* methanolic extract after hydrolysis with sulphuric acid for 1 hour at 60 °C is shown in Figure 2.27.

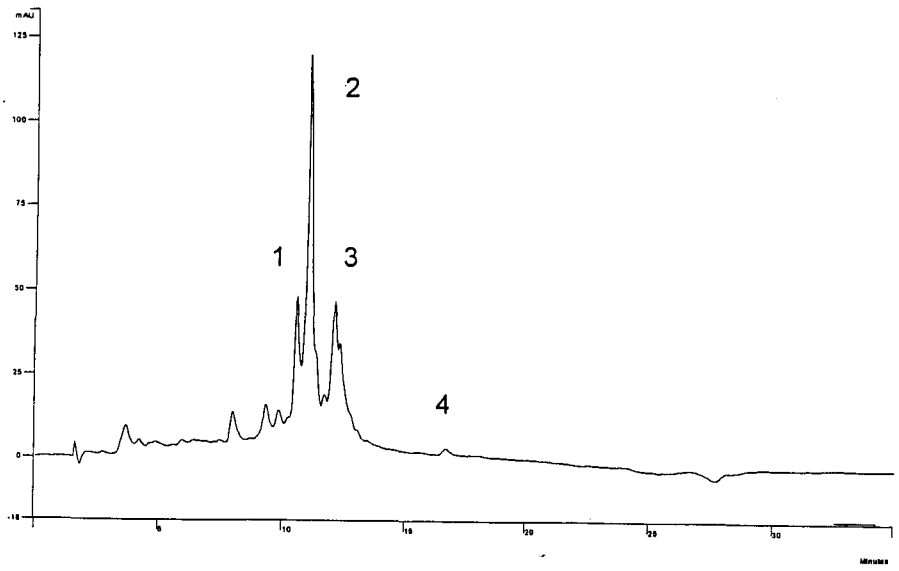


Figure 2.26 HPLC chromatogram of *C. rossii* methanolic extract (non-hydrolysed).

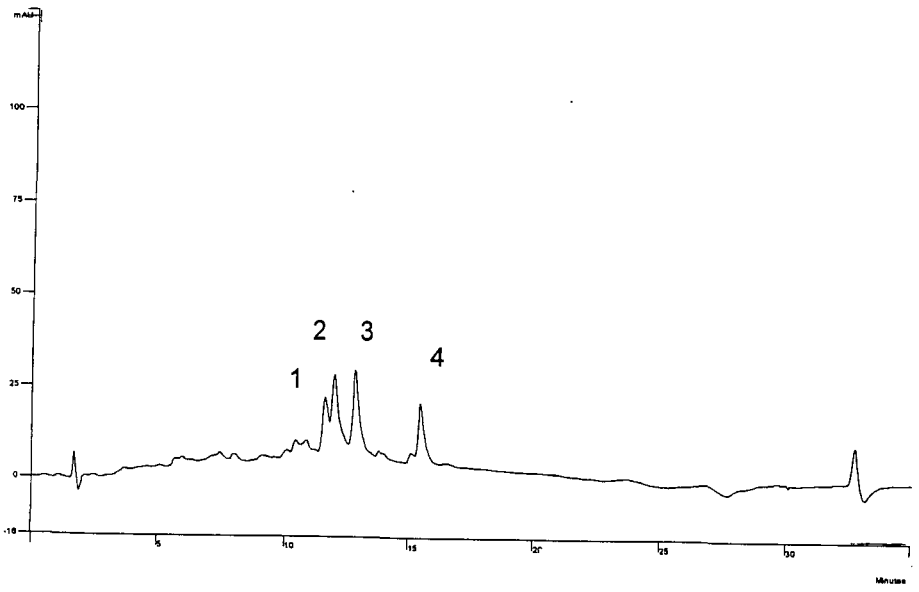


Figure 2.27 HPLC chromatogram of *C. rossii* methanolic extract (hydrolysed).

From Figure 2.26 the peaks 1, 2 and 3 represent the flavonoids. In Figure 2.27 we can see that the area of peaks 1, 2 and 3 has decreased and the area of peak 4 has increased after treatment of the sample with sulphuric acid. Figure 2.28 shows the UV spectrum of Peak 4 (at retention time 15.86 minutes), which had typical flavonoid UV absorbance but had different absorbance from the other flavonoids (UV maxima at 261 nm and 377 nm). Figure 2.29 shows the UV absorbance of peak 2 with different absorbance maxima (260 nm and 366 nm). This suggests that compounds 1, 2 and 3 which are in the form of glycosides undergo hydrolysis and give compound 4. LC-MS confirmed that the compound responsible for peak 4 had a MW of 346, consistent with the aglycone moiety of the original flavonoid glycosides. Hydrolysis by sulphuric acid for one hour at 60 °C resulted in hydrolysis of about 25% of the glycosides. Hydrolysis using hydrochloric acid appeared to destroy all the flavonoids and with acetic acid the hydrolysis process was slow. From the results of the above experiment it is suggested that hydrolysis of *C. rossii* methanol extract with sulphuric acid for one hour at 60 °C would yield an aglycone moiety, which can be further analysed for structural determination.

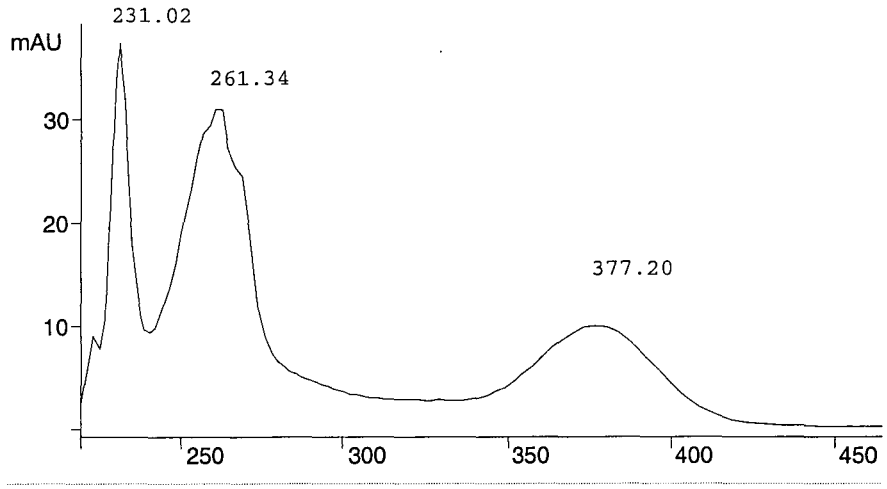


Figure 2.28 UV spectrum of *C. rossii* methanolic extract (hydrolysed) peak 4 with retention time 15.86 minutes.

Figure 2.29 UV spectrum of *C. rossii* methanolic extract (non-hydrolysed) peak 2 with retention time 11.09 minutes.

2.5.7 GC-MS

Figure 2.30 shows the GC-MS chromatogram of *Carpobrotus edulis* volatiles from freshly prepared juice sampled by SPME. Table 2.13 shows the major peaks and the corresponding chemical compounds identified by GC-MS. Figure 2.31 shows the volatile constituents of *C. edulis,* sampled by using the 85 μm Supelco Polyacrylate fibre and Table 2.14 shows the major peaks and corresponding chemical compounds identified by GC-MS.

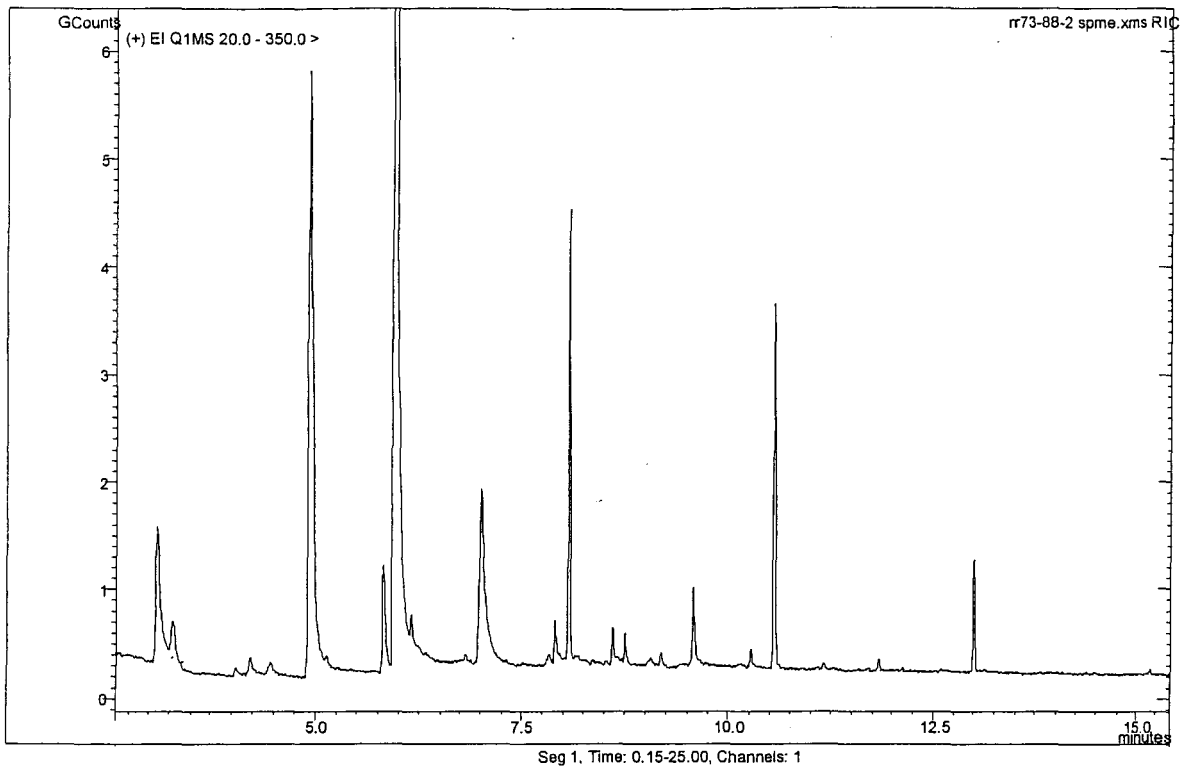


Figure 2.30 GC chromatogram of *C. edulis* volatile compounds sampled using Supelco 75 µm Carboxen-PDMS SPME fibres.

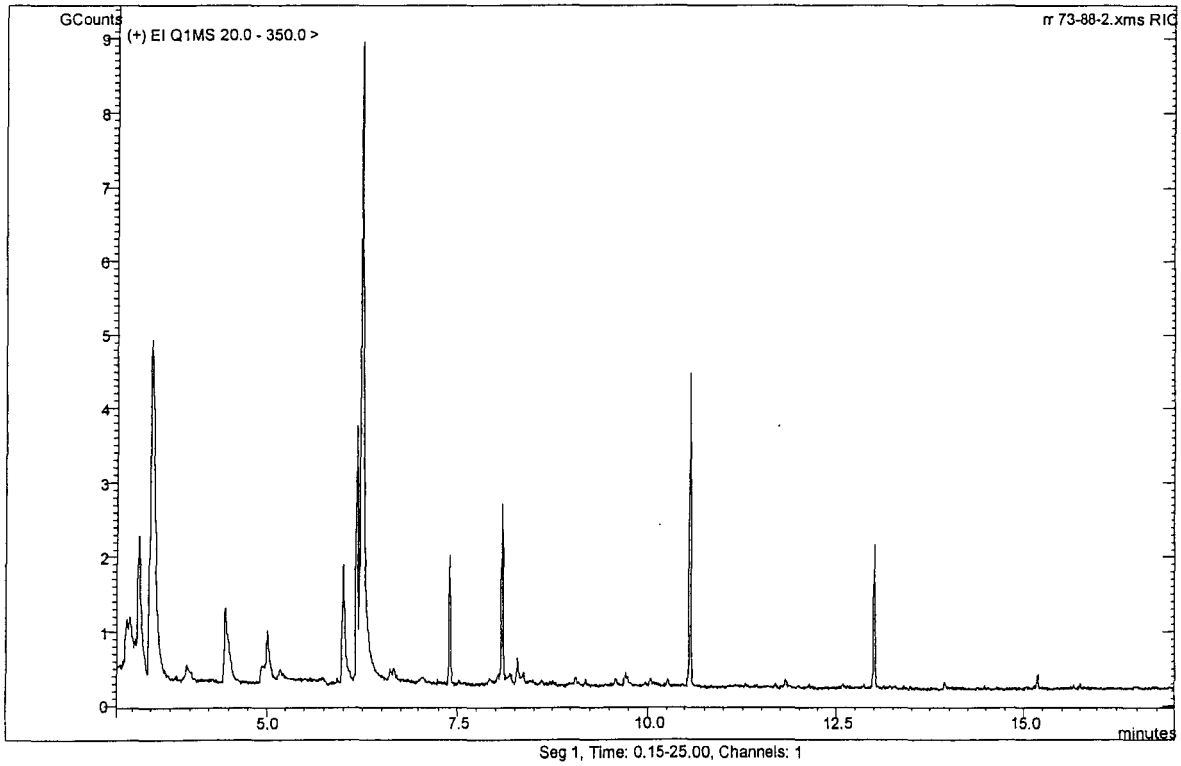


Figure 2.31 SPME chromatogram of *C. edulis* volatile compounds using 85 µm Supelco Polyacrylate fiber.

Table 2.13 Volatile compounds with their corresponding peaks by SPME (Supelco 75 μm Carboxen-PDMS fibres) of *Carpobrotus* species.

Retention time (min)	% peak area C. edulis	*C. rossii*	*C. aequilaterus*	Identified compounds using NIST library search
3.09	6.1	2.4	6.0	1-Peten-3-one
4.91	27.8	10.8	9.8	3-Hexenal
4.94	21.1	8.2	6.3	Hexenal
5.80	5.8	2.2	0.1	2-Hexenal
5.94	17.7	68.7	10.8	2-Hexenal
6.14	-	-	-	Contaminants
6.99	13.6	0.6	1.7	2,4-Hexadienal
7.88	2.0	5.3	61.7	4-oxo-Z-hexenal
8.07	-	-	-	Contaminants
8.59	1.4	0.8	0.3	3-Hexen-1-ol acetate
8.75	1.4	0.5	0.3	2-Hexen-1ol acetate
9.56	3.0	0.5	3.0	2,2-Dimethyl-3-heptanone
10.54	-	-	-	Contaminants
12.99	-	-	-	Contaminants

Table 2.14 Volatile compounds with their corresponding peaks by SPME (85 μm Supelco Polyacrylate fibre) of *Carpobrotus* species.

Retention time (min)	% peak area			Identified compounds using NIST library
	C. edulis	*C. rossii*	*C. aequilaterus*	
4.45	11.1	3.5	5.8	1-Pentenol
5.01	6.7	0.9	2.3	Hexanal
6.17	16.2	2.4	11.9	3-Hexen-1-ol
6.23	64.2	79.8	73.5	2-Hexen-1-ol
6.64	0.6	12.3	5.2	3-Hexenol
7.39	-	-	-	Contaminants
8.08		-	-	Contaminants
8.28	0.6	0.6	0.6	3-Octanone
9.68	0.3	0.3	0.3	1-Nonanal
10.55	-	-	-	Contaminants
11.78	0.3	0.3	0.3	Decanal
12.99	-	-	-	Contaminants

The three plant species contained similar kinds of volatile constituents like pentenone and hexenal, when fresh juices were analysed. The profile of compounds differs between species. For example *C. rossii* has 69% 2-hexenal compared with less than 20% in the other species. These compounds are typically found in most green plants. So-called grassy aldehydes are responsible for the smell of cut grass. There were no terpenes present, only simple straight chain compounds were present. When the juices were left at room temperature for a week and then analysed there was a difference in the volatile constituents; the aldehydes were converted to alcohols. Hexenal was converted to hexenol, pentenone to pentenol. This suggests there was a change in volatile constituents due to the oxidase enzymes in plant juices or maybe due to fermentation by microbes.

2.5.8 Analysis of carbohydrate content in *Carpobrotus* species by using HPLC with ELSD detection

The three *Carpobrotus* species were analysed by HPLC-ELSD for their carbohydrate content using a Prevail ES carbohydrate column. Figure 2.32 shows the HPLC-ELSD chromatogram of oligofructan carbohydrate standards in an aqueous onion extract. The extract was spiked with a mixture of kestose (DP3), nystose (DP4), and 1-β-fructofuranosyl-D-nystose (DP5) at 5.1 mg/ml, 8.1 mg/ml and 1.5 mg/ml respectively to demonstrate resolution of oligo-fructans which were not evident as a major constituent in the *Carpobrotus* extracts. The ELSD chromatogram of *C. edulis, C. rossii* and *C. aequilaterus* plant juices are shown in Figures 2.33, 2.34 and 2.35 respectively.

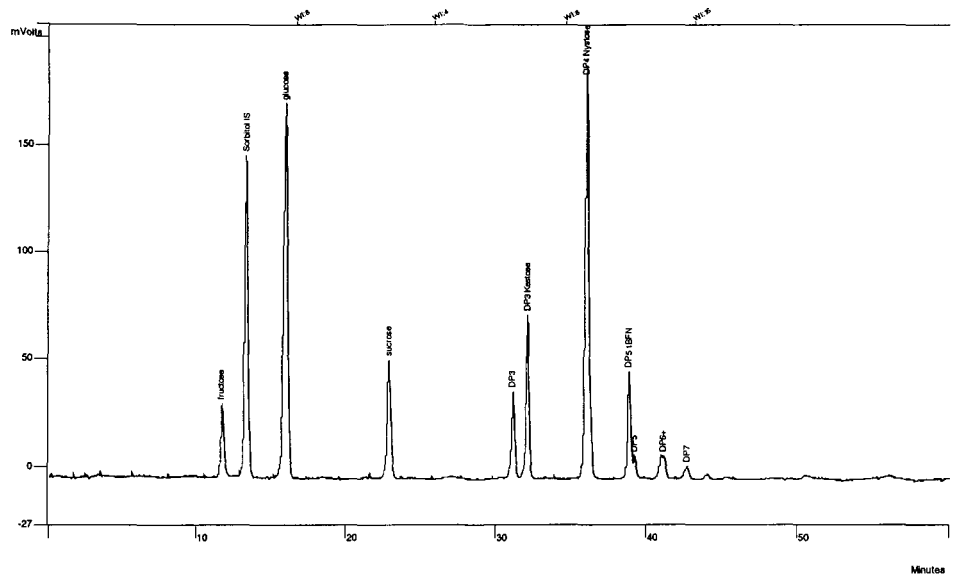


Figure 2.32 HPLC-ELSD chromatogram of carbohydrate standards in an aqueous onion extract.

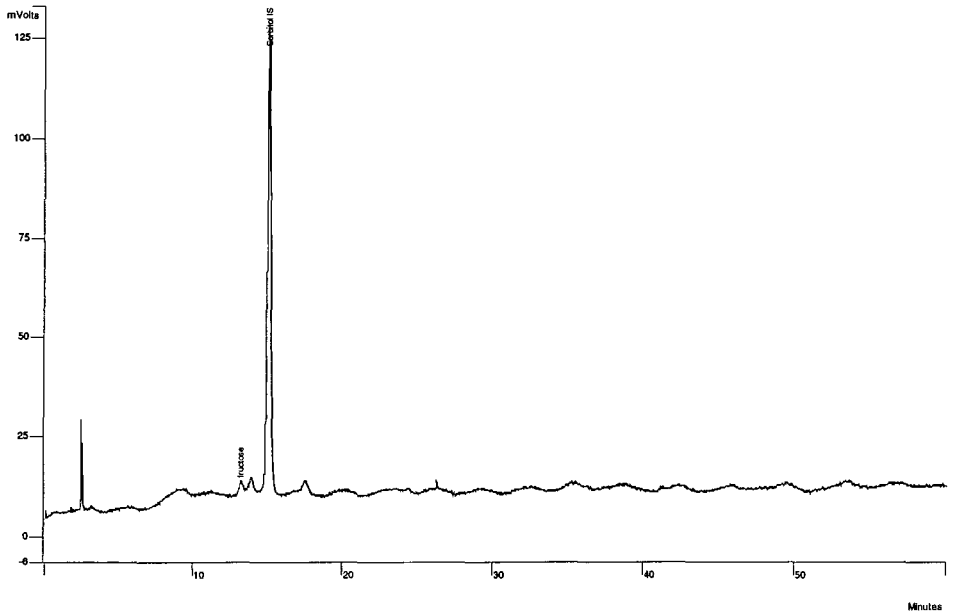


Figure 2.33 HPLC-ELSD chromatogram of carbohydrates in *C. edulis* plant juice

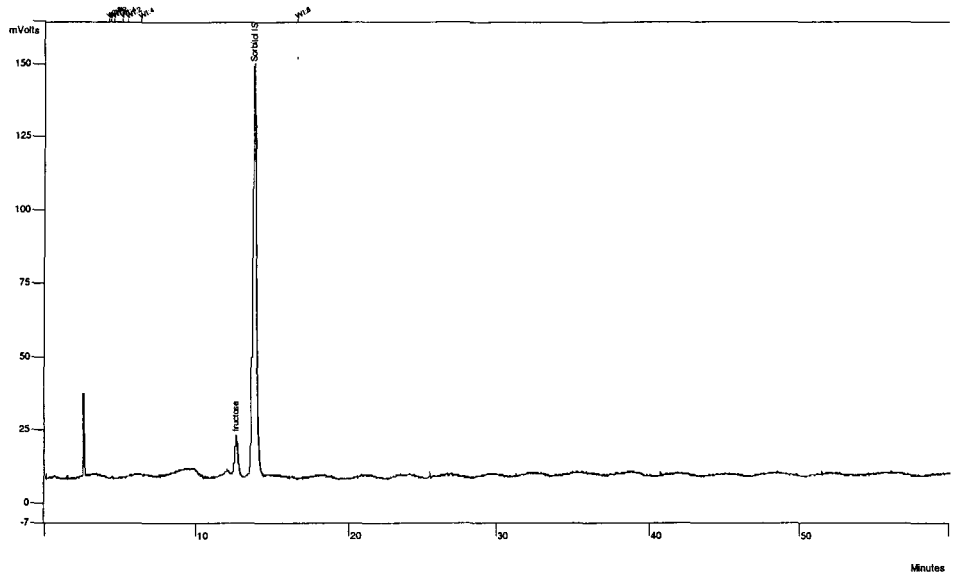


Figure 2.34 HPLC-ELSD chromatogram of carbohydrates in *C. rossii* plant juice

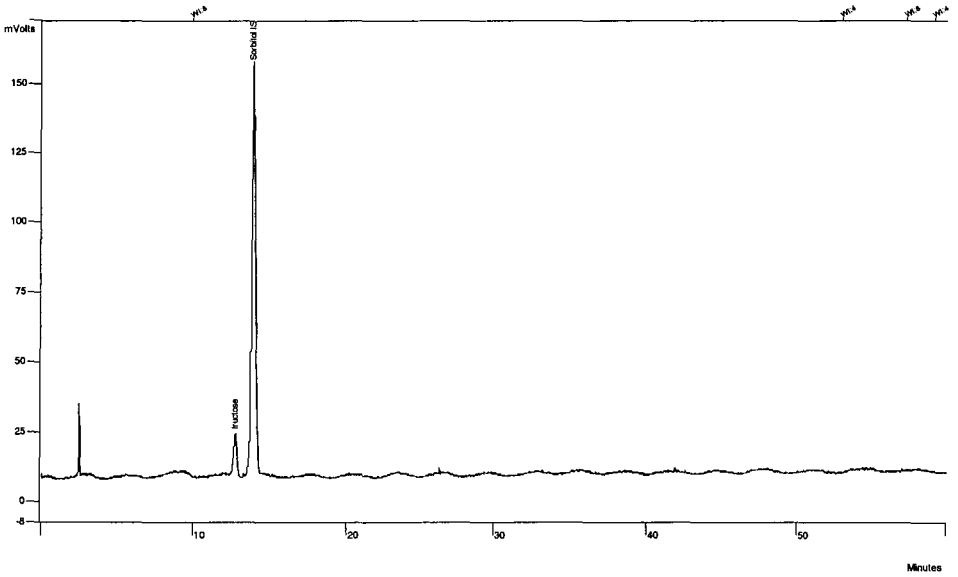


Figure 2.35 HPLC-ELSD chromatogram of carbohydrates in *C. aequilaterus* plant juice.

Identification and approximate quantitation of simple sugar content was made on the basis of retention time only. *C. rossii* and *C. aequilaterus* were found to have higher fructose content than *C. edulis*. A peak consistent with the retention time of glucose and another unidentified peak were also found in *C. edulis* that did not match the sugars in the carbohydrate standard solutions. The fructose content was estimated as 6.8 mg/ml, 2.8 mg/ml, and 1.2 mg/ml for *C. aequilaterus, C. rossii*, and *C. edulis* respectively.

3. Antioxidant capacity of *Carpobrotus* species

3.1 Objective

From the previous chemistry study on the *Carpobrotus* species in chapter 2, we found that the flavonoids and polyphenolics were major chemical constituents. Given the polyphenolics and flavonoids have antioxidant activity, this study was conducted to evaluate the antioxidant properties of *Carpobrotus* species plant juice and methanolic extracts. Ascorbic acid and phenolic content were also measured to assist in characterisation of the antioxidants. The β-carotene bleaching method, DPPH free radical scavenging assay and FRAP assay were used to obtain a measure of antioxidant activity.

3.2 Materials

3.2.1 Chemicals

The chemicals used were rutin hydrate, gallic acid, ascorbic acid, DPPH (2,2-diphenly-2-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), ferrous sulphate, β-carotene, linoleic acid, trichloroacetic acid (Sigma-Aldrich, Sydney, Australia), methanol AR grade (BDH/Merck, Kilsyth, Victoria, Australia), sodium acetate (BDH, England), acetic acid (Ajax, Australia), hydrochloric acid (Biolab, Victoria, Australia), ferric chloride (BDH, Poole, England), Tween 20 (Polymer corporation Pty Ltd, Sydney, Australia), 2,4-dinitrophenylhydrazine (Ajax, Australia) and sodium carbonate anhydrous (Ajax, Australia) .

3.2.2 Equipment

Polystyrene cuvettes (Kartell, Italy), were used in UV-Visible spectrophotometer (UV Mini 1240, Shimadzu, Sydney, Australia). Microtitre plate reader (Tecan, GENios, Austria) was used in the FRAP assay. Microtitre plate reader (Model 680, Bio-Rad, Sydney, Australia) was used in the DPPH assay. Microtitre plates used were from Corning Inc. New york, US. Liquid chromatography - mass spectrometry (LC-MS) was performed using a Waters Alliance 2690 High performance liquid chromatograph (HPLC) and Waters 996 Photo Diode Array (PDA) detector coupled to a Finningan LCQ ion trap mass spectrometer. The column used was Waters Nova_Pak C18 (150 mm x 3.9 mm).

3.3 Methods

3.3.1 Plant collection

Three samples of each of three different species of *Carpobrotus* were collected from different places in Tasmania as detailed in Table 2.1. Juices was extracted from these nine plants as previously described.

3.3.2 Ascorbic acid contents of plant juices

3.3.2.1 Background

Many chemical methods for the determination of reduced ascorbic acid are based upon its reducing properties. The assay chosen was based on the reaction of

dehydroascorbic acid with 2,4-dinitrophenylhydrazine to form a coloured product, which is measured at 520 nm. This assay is not dependent on the reducing properties of ascorbate, but depends on the formation of a coloured complex between dehydroascorbic acid and 2,4-dinitrophenlyhydrazine. (Schlesier *et al.*, 2002).

3.3.2.2 Reagent preparation

Aqueous ascorbic acid standards were prepared in water from a stock solution of 80 mg/100 ml water. Two-fold dilutions were made in the range of 125 to15.6 μg/ml. 2, 4-Dinitrophenylhydrazine reagent was prepared by dissolving 2.0 g in 100 ml of 9 N sulphuric acid. After letting the reagents stand overnight, the solution was filtered a through Whatman No. 42 filter paper by using a funnel (Schaffert, and Kingsley, 1955). A 4% w/v trichloroacetic acid solution was prepared in water. The *Carpobrotus* species plant juices (CeJ1, CeJ2, CeJ3, CrJ1, CrJ2, CrJ3, CaJ1, CaJ2 and CaJ3) were prepared by 20 times dilution with water.

3.3.2.3 Ascorbic acid assay method

In this assay 200 μl of the solution containing ascorbic acid were mixed with 300 μl of trichloroacetic acid solution. An aliquot of this mixture (300 μl) was mixed with 100 μl of 2,4-dinitrophenylhydrazine reagent in Eppendorf ®vials and heated for 1 hour at 60 °C on a heating block. Then the solution mixture was cooled in an ice bath and 400 μl of sulphuric acid were added and mixed vigorously. After 20 minutes in the dark, the solution mixture was transferred to low volume disposable polystyrene cuvettes. The optical absorbance of the samples was measured at 520 nm (Schlesier *et al.*, 2002). A standard curve of absorbance versus concentration of

ascorbic acid was plotted, using the 2,4-dinitrophenlyhydrazine method. The concentration of ascorbic acid in plant juices was calculated from the standard curve by interpolation. The concentration of ascorbic acid obtained was then multiplied by the dilution factor to give the actual concentration of ascorbic acid in each plant juice sample. The dilution factor was 20 for all plant juices.

The accuracy of the method was calculated, but since the experiment was conducted in cuvettes only one reading was taken, so accuracy was calculated over the range of the calibration curve taking into account of all points on the calibration curve. Assay performance was assessed using the correlation coefficient of the calibration curve. Accuracy (observed – expected/expected x 100) was also calculated.

3.3.3 β-Carotene bleaching assay

3.3.3.1 β-carotene bleaching assay background

β-carotene is the predominant carotenoid in plants. Carotenoids have an extended system of conjugated double bonds, which is responsible for their antioxidant activity. The β-Carotene bleaching method developed by Taga *et al.,* (1984) estimates the relative ability of antioxidant compounds in the plant extracts to scavenge the radical of linoleic acid peroxide that oxidises β-carotene in the emulsion phase (Kaur and Kapoor, 2001). The β-carotene bleaching method used in this project was modified for use with microtitre plates (Fukomoto and Mazza, 2000).

3.3.3.2 β-Carotene reagent preparation

Rutin standards were prepared from a stock solution of 120 mg/100 ml in methanol. Dilutions were made with methanol to 200, 100, 50, and 25 μg/ml. Gallic acid standards were prepared from a stock solution of 40 mg/100 ml in methanol. Dilutions were made to 40, 20, 10 and 5 μg/ml from the stock solution with methanol. Ascorbic acid standards were prepared from a stock solution of 120 mg/100 ml in water. Dilutions were made with water to 1200, 600, 300, 150, 75, 37.5, and 18.8 μg/ml.

All *Carpobrotus* species plant juices were prepared as 1-, 2-, 4-, and 8-fold dilutions with methanol.

3.3.3.3 β-Carotene assay Method

A mixture of 1 ml of β-carotene solution (2 mg in 10 ml of dichloromethane), 5 μl of linoleic acid and 40 μl of Tween 20 was prepared. The mixture was vortex mixed and transferred to a 50 ml round bottom flask. Dichloromethane was removed using a rotary evaporator. Oxygenated distilled water (10 ml prepared by bubbling oxygen into the pure distilled water) was added to the β-carotene emulsion and then vortex mixed to form a clear solution. Samples of plant juice solutions (20 μl) and β-carotene emulsion (250 μl) were added to a well in a flat bottom microtitre plate. Samples were prepared in triplicate for each plant juice sample. The microtitre plate was read at 490 nm before starting the incubation to obtain the initial absorbance. The microtitre plate was incubated at 50 °C for 2 hours after which absorbances were read at 490 nm.

The results were calculated as antioxidant activity coefficient (AAC) based on the formula below (Mallet *et al.*, 1993).

$$AAC = \frac{A_{S(120)} - A_{C(120)}}{A_{C(0)} - A_{C(120)}} \times 1000 \quad (1)$$

Where $A_{S(120)}$ was the absorbance of the antioxidant sample at time t = 120 min, $A_{C(120)}$ the absorbance of the control at t = 120 min and $A_{C(0)}$ the absorbance of the control at t = 0.

The AAC was calculated for the standard antioxidants and plant juices by using the above formula (1). The calibration curve of AAC versus concentration was constructed for standard antioxidants. From the calibration curves the standard equivalents were calculated for each of the juices by interpolation. Thus rutin, gallic acid and ascorbic acid equivalents were calculated for all plant juices.

β-Carotene assay performance was assessed using the correlation coefficient of the calibration curve of all three standards gallic acid, rutin and ascorbic acid. Accuracy (observed – expected)/expected x 100 (n=3) and precision were reported as percent relative standard deviation (% RSD; n=3).

3.3.4 DPPH radical scavenging assay

3.3.4.1 DPPH radical scavenging assay background

The radical scavenging activity of plant extracts against the stable DPPH radical is determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced and changes in colour from deep violet to light yellow. The decrease in absorbance at or near 515 nm can be measured spectrophotometrically. The method used to measure the radical scavenging activity of extracts was based on the method of Brand-Williams *et al.*, (1995). The method was modified in order to perform the assay in microtitre plates.

3.3.4.2 DPPH radical scavenging assay reagent preparation

DPPH solution was prepared by dissolving approximately 5 mg of DPPH in 100 ml of methanol. The absorbance of 250 μl in a microtitre plate well was measured and the concentration adjusted with DPPH to give an initial absorbance of 1.5 at 490 nm. The solution, after preparation, was protected from light by covering the flask with aluminum foil.

Rutin standards were prepared from a stock solution of 120 mg/100 ml in methanol. Dilutions were made to 600, 300, 150, 75, 38, 19, and 9.5 μg/ml. Gallic acid standards were prepared from a stock solution of 20 mg/100 ml in methanol. Dilutions were made to 100, 50, 25, 12.5, 6.2, 3.1, and 1.6 μg/ml from the stock solution with methanol. Ascorbic acid standards were prepared from a stock solution of 80 mg/100 ml in water. Dilutions were made to 400, 200, 100, 50, 25, 12.5, and 6.25 μg/ml.

The *Carpobrotus* species juices were prepared by dilution to 4, -8, -16, -32, -64, -128, -256 and -512 fold with methanol.

3.3.4.3 DPPH radical scavenging assay method

The major modification of the published method was to reduce the quantities of the reagents used accordingly, in order to perform the assay in microtitre plates. Aliquots (12 μl) of different concentrations of standards (rutin, gallic acid and ascorbic acid), and controls (water and methanol) were pipetted into wells of the microtitre plate in triplicate. Aliquots (12 μl) of the plant juice dilutions were prepared and were pipetted into wells of the microtitre plate in triplicate. Then 250 μl of DPPH solution was rapidly pipetted into each well. The microtitre plate was incubated in the dark for 30 minutes and read at 490 nm.

Absorbance versus concentration calibration curves were constructed for the standards rutin, gallic acid and ascorbic acid (Figures 3.5, 3.6 and 3.7). The maximum absorbance value was taken as the average of three control determinations (no antioxidant added). In this example (rutin) the maximum absorbance was 1.40. The minimum absorbance value was taken as the average of three determinations containing excess antioxidant. In this example (rutin) the minimum absorbance was 0.20. The midpoint between the minimum and maximum absorbances was calculated (0.80). For each antioxidant standard the concentration of antioxidant corresponding to the absorbance midpoint was determined by interpolation from the standard curve. This concentration was considered to be the EC_{50} of that antioxidant under the described assay conditions. The results are summarised in Table 3.6.

For each plant juice a graph of absorbance versus inverse of dilution factor (concentration) was plotted. An example of such a plot is shown in Figure 3.8. The EC_{50} was determined in a similar way as with the antioxidant standards, to give a concentration expressed as a dilution of the original plant juice. In the example (CeJ2) this dilution value was 101. These values were multiplied by the EC_{50} values of the standard antioxidants (Table 3.6). This gave the antioxidant value of the original juice expressed as an equivalent concentration of antioxidant standard. In the example (CeJ2) the juice had DPPH antioxidant power equivalent to 32 mg/ml of rutin, 6.5 mg/ml of gallic acid and 21 mg/ml of ascorbic acid. The results for all the *Carpobrotus* species juice samples are summarised in Table 3.7.

Plant juice ascorbic acid concentration was calculated for each plant juice by interpolation from the ascorbic acid standard curve. The results are summarised in Table 3.2. The ascorbic acid concentrations were used to calculate the fraction of DPPH antioxidant activity due to ascorbic acid in each plant juice. Thus the fraction of DPPH antioxidant activity due to antioxidants other than ascorbic acid was calculated for each of the plant juices.

DPPH assay performance was assessed using the correlation coefficient of calibration curve of all three standards gallic acid, rutin and ascorbic acid. Accuracy (observed – expected)/expected x 100 (n=3) and precision were reported as percent relative standard deviation (% RSD; n=3) are shown in Table 3.5.

3.3.5 FRAP assay: The Ferric Reducing and Antioxidant Power

3.3.5.1 Background of FRAP assay

The ferric reducing and antioxidant power (FRAP) assay, originally termed the ferric reducing ability of plasma assay, is one of the novel methods of assessing antioxidant power.

This method involves reduction of the ferric-tripyridyltriazene (Fe^{3+} – TPZP) complex to the ferrous (Fe^{2+}) form at low pH to give an intense blue colour having an absorption maximum at 593 nm. FRAP values are obtained by comparing the absorbance changes at 593 nm in the test reaction mixture with the absorbance of solutions containing ferrous ions in known concentrations (Benzie and Strain, 1996). Absorbance changes are linear over a wide concentration range with antioxidant mixtures. The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible and the procedure is straightforward and speedy.

3.3.5.2 FRAP assay reagent preparation

Acetate buffer (0.30 M, pH 3.6) was prepared using 3.1 g of sodium acetate and 16 ml acetic acid per litre of buffer solution. TPTZ solution 10 mM was prepared using 30 μg of TPTZ in 10 ml of 40 mM HCl . Ferric chloride solution (20 mM) was prepared using 5.4 g of $FeCl_3.6H_2O$ in 1.0 L of water. Working FRAP reagent was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml ferric chloride solution.

3.3.5.3 FRAP assay sample preparation

Aqueous solutions of known ferrous (Fe^{2+}) ion concentration using ferrous sulphate ($FeSO_4$ .7 H_2O) were prepared in the range of 100 – 1000 μmol/l and were used for calibration. Rutin standards were prepared from a stock solution of 120 mg/100 ml in methanol and two-fold dilutions were made in the range of 600 to 9.4 μg/ml.

Gallic acid standards were prepared from a stock solution of 20 mg/100 ml in methanol and two-fold dilutions were made in the range of 100 to 1.6 μg/ml.

Ascorbic acid standards were prepared from a stock solution of 80 mg/100 ml in water and two-fold dilutions were made in the range of 400 to 6.3 μg/ml.

The *Carpobrotus* species plant juices (CeJ1, CeJ2, CeJ3, CaJ1, CaJ2, CaJ3, CrJ1, CrJ2 and CrJ3) were prepared by diluting 4, 8, 16, 32, 64, 128, 256 and 512 times with water.

3.3.5.4 FRAP assay method

The reaction was carried out using a microtitre plate. Distilled water (30μl) and antioxidant sample solution (10 μl) were pipetted into each microtitre plate well. FRAP solution (200μl) was added, mixed for 10 s and the absorbance was measured at 590 nm after 8 min using a Tecan microtitre plate reader (Schlesier *et al.*, 2002).

The ferrous ion concentration in μmol/l (FRAP number) was plotted against absorbance at 590 nm to give a standard curve for FRAP analysis, shown in Figure 3.9. The FRAP numbers were determined for each of the standard antioxidant solutions by interpolation from the standard ferrous sulphate curve.

A graph of standard antioxidant concentration versus FRAP number was plotted for each antioxidant gallic acid, rutin and ascorbic acid (Figures 3.10, 3.11 and 3.12). The concentration of standard antioxidant at 1000 FRAP number was taken as the FRAP 1000 value of that antioxidant. From Figure 3.10 the FRAP 1000 value for gallic acid was 50 μg/ml. Concentrations of different standard antioxidants at FRAP 1000 are given in Table 3.9.

The FRAP 1000 value of plant juice samples were determined in a similar way to the antioxidant standards, giving a concentration expressed as a dilution of the original plant juice. These values were then multiplied by the FRAP 1000 values of the standard antioxidants. This gave the antioxidant value of the original juice expressed as an equivalent concentration of antioxidant standard. In the example given below, CeJ2 plant juice had antioxidant potential equivalent to 38 mg/ml of rutin, 5.4 mg/ml of gallic acid and 12 mg/ml of ascorbic acid. The results for all the *Carpobrotus* juice samples are summarised in Table 3.10.

The ascorbic acid concentrations given in the Table 3.2 were used to calculate the fraction of FRAP antioxidant activity due to ascorbic acid in the plant juice. Thus the fraction of FRAP antioxidant activity due to antioxidants other than ascorbic acid was calculated for each of the plant juices. The results are summarised in Table 3.10.

FRAP assay performance was assessed using the correlation coefficient of calibration curve of all three standards gallic acid, rutin and ascorbic acid, accuracy (observed – expected)/expected x 100 (n=3) and precision were reported as percent relative standard deviation (% RSD ; n=3). The results are shown in Table 3.8

3.3.6 Total phenolic content

3.3.6.1 Background of total phenolics

Total phenolics in *Carpobrotus* species were determined using Folin-Ciocalteau reagent. This method is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. The green-blue complex after oxidation was measured at 650nm, using gallic acid monohydrate as a standard (Schlesier *et al.*, 2002). Results are expressed as gallic acid equivalents.

3.3.6.2 Total phenolics assay reagent preparation

Gallic acid standard solutions were prepared from a stock solution containing 30 mg gallic acid in 100 ml ethanol. Dilutions were made to give 30, 15, 5.0, 2.0 and 1.0 mg/ml in ethanol. Sodium carbonate solution was prepared by dissolving 75 g of sodium carbonate in 1000 ml of water.

3.3.6.3 Total phenolics assay sample preparation

Three species of *Carpobrotus* were assayed for total phenolics. *Carpobrotus aequilaterus* (CaJ1, CaJ2 and CaJ3), *C. rossii* (CrJ1, CrJ2 and CrJ3) and *C. edulis* (CeJ2) plant juices were diluted 10 times with methanol. *Carpobrotus edulis* (CeJ1 and CeJ2) plant juices were diluted 20 times with methanol.

3.3.6.4 Total phenolics assay method

The assay was adapted to be performed on microtitre plates. Each plant juice was analysed at a single dilution in triplicate. In each well of the flat bottom microtitre plate, 20 μl of diluted plant juice sample or antioxidant standard, 100 μl of Folin-Ciocalteu reagent and 100 μl of sodium carbonate solution were added. Then the microtitre plate was incubated for 60 mins. Absorbance was measured at 650 nm using a microtitre plate reader (Bio-Rad, Sydney, Australia).

3.3.7 Analysis of flavonoids having antioxidant activity in *Carpobrotus edulis* plant juice using LC-MS

Carpobrotus rossii plant juice was subjected to gel-permeation chromatography for separation of the antioxidants. Sephadex LH20 gel was swelled overnight in 80% ethanol. A 50 ml burette was filled with 18 ml gel bed volume. Initial solvent used was 80% ethanol. *C. rossii* plant juice (4 ml) was applied to the column and eluted with 80% ethanol, with 1 ml fractions collected in Eppendorf ® vials. After collecting 40 fractions, 50% acetone/water was used to elute and 1 ml fractions were collected. Fractions obtained from 80% ethanol (40 fractions) and 50% acetone (40 fractions) were checked for antioxidant activity using the DPPH method. The seventeenth acetone fraction was found to have maximum antioxidant activity. This acetone fraction was analysed using LC-MS. The column used was Waters Nova-Pak C18 (150 mm x 3.9 mm) with flow rate 0.8 ml/min, 10 μl injection volume. Solvents used were 2% acetic acid in methanol (A) and 2% acetic acid in water (B). A linear gradient elution profile was followed from 10% A and 90% B to 100% A over 40 minutes.

3.4 Results

3.4.1 Ascorbic acid assay results

The calibration curve is shown in Figure 3.1. The results of the ascorbic acid assay for *Carpobrotus* species plant juices are summarised in Table 3.1. The accuracy of the assay was found to be ± 1.48%.

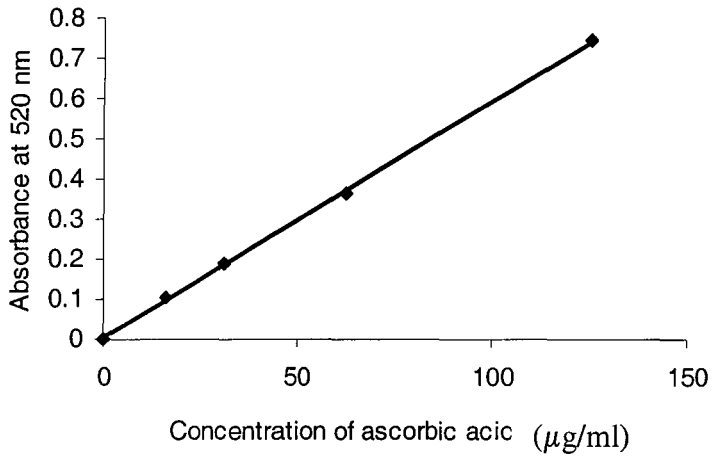


Figure 3.1 Standard curve of ascorbic acid with absorbance versus concentration.

Table 3.1 Ascorbic acid concentration in different *Carpobrotus* species plant juice.

Carpobrotus species plant juices	Absorbance	Concentration of ascorbic acid in 20 times diluted plant juice (µg/ml)	Concentration of ascorbic acid in plant juice (mg/ml)
Ce[a]J1	0.523	87.9	1.76
CeJ2	0.464	77.9	1.56
CeJ3	0.148	24.4	0.49
Cr[b]J1	0.305	51.1	1.02
CrJ2	0.254	42.4	0.85
CrJ3	0.177	29.3	0.59
Ca[c]J1	0.322	53.9	1.08
CaJ2	0.219	36.6	0.73
CaJ2	0.257	42.9	0.86

[a]Ce is *C. edulis*, [b]Cr is *C. rossii*, [c]Ca is *C. aequilaterus*.

3.4.2 β-Carotene assay results

The antioxidant activity of plant juices using the β-carotene assay is shown in Table 3.3 and calibration curves for rutin, gallic acid and ascorbic acid are shown in Figures 3.2, 3.3 and 3.4 respectively. The accuracy and precision of the β-carotene assay was calculated and results are shown in Table 3.2.

Table 3.2 Accuracy and precision of β-carotene assay.

β-Carotene		Precision %RSD (n=3)	Accuracy (%) (n=3)
Rutin	lower concentration	0.91	-0.05
	higher concentration	9.08	0.20
Gallic acid	lower concentration	5.38	< 0.01
	higher concentration	0.37	-0.05
Ascorbic acid	lower concentration	7.90	-0.86
	higher concentration	19.4	< 0.01

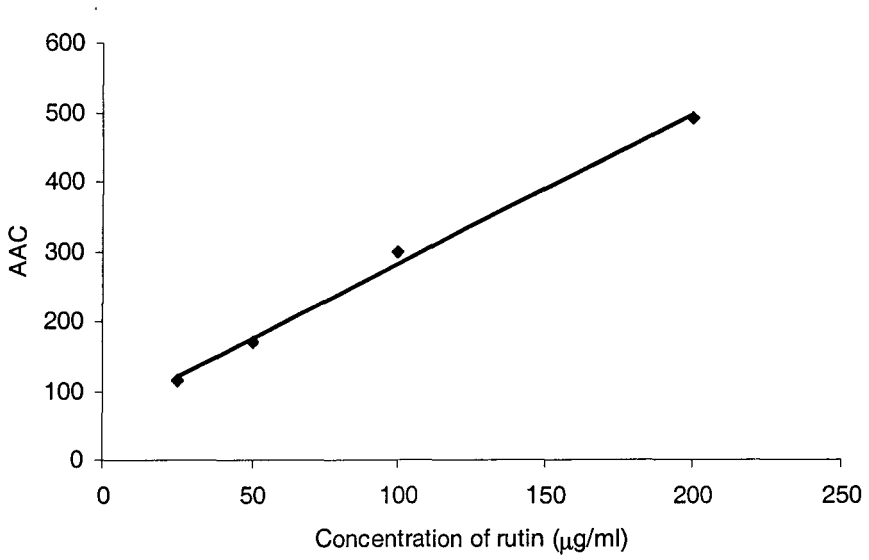


Figure 3.2 Standard curve of rutin in the β-carotene assay AAC (r^2= 0.992).

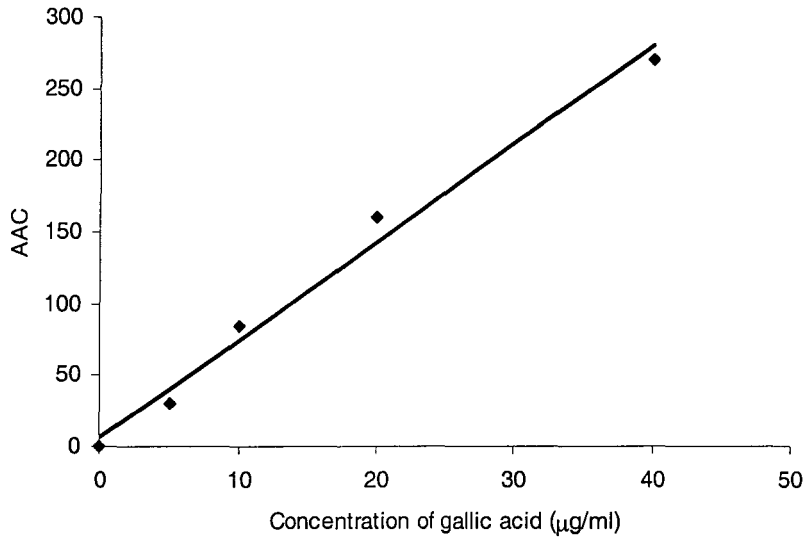


Figure 3.3 Standard curve of gallic acid in the β-carotene assay AAC (r^2= 0.984).

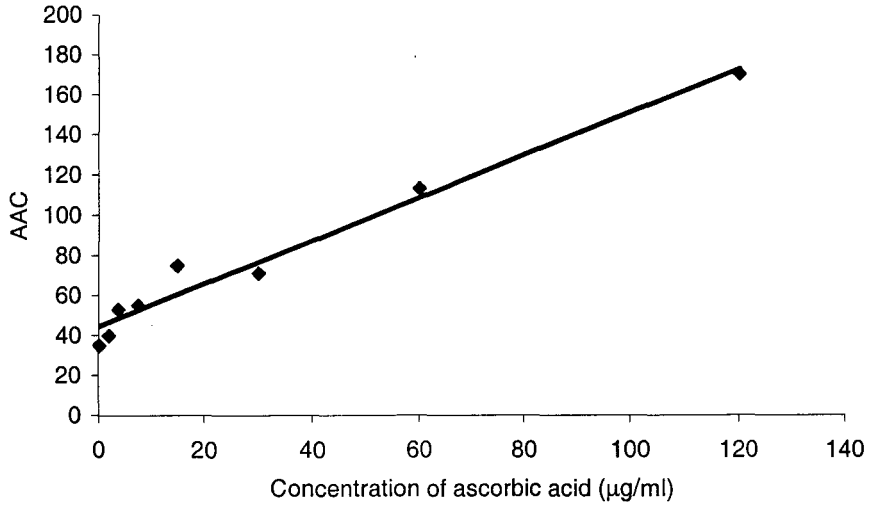


Figure 3.4 Standard curve of ascorbic acid in the β-carotene assay AAC (r^2= 0.973).

Table 3.3 Antioxidant activity by the beta-carotene assay of all plant juices of *Carpobrotus* species.

Carpobrotus species plant juice	Number of Dilutions of plant juice	AAC	Total rutin equivalent (mg/ml)	Total gallic acid equivalent (mg/ml)	Total ascorbic acid equivalent (mg/ml)
Ce[a]J1	64	413.6	9.7	2.5	17.6
CeJ2	64	330.7	7.3	2.0	13.6
CeJ3	16	111.9	0.3	0.2	0.8
Cr[b]J1	64	324.8	7.2	1.9	13.3
CrJ2	64	277.2	5.8	1.7	11.2
CrJ3	64	174.8	2.9	1.0	6.2
Ca[c]J1	64	283.6	6.0	1.7	11.4
CaJ2	64	257.3	5.3	1.5	10.1
CaJ3	64	367.4	8.4	2.2	15.3

[a]Ce is *C. edulis,* [b]Cr is *C. rossii,* [c] Ca is *C. aequilaterus.*

3.4.3 DPPH radical scavenging assay results

The antioxidant activity of *Carpobrotus* species plant juice using the DPPH assay are summarised in Table 3.6. The calibration curves of rutin, gallic acid and ascorbic acid are shown in Figures 3.5, 3.6 and 3.7. A graph of absorbance versus inverse of dilution factor (concentration) for CeJ2 is shown in Figure 3.8. The accuracy and precision of the DPPH assay was calculated and results are shown in Table 3.4.

Table 3.4 Accuracy and precision of DPPH assay.

DPPH assay		Precision %RSD (n=3)	Accuracy (%) (n=3)
Rutin	lower concentration	7.56	0.09
	higher concentration	0.15	-0.02
Gallic acid	lower concentration	5.02	0.08
	higher concentration	1.13	< 0.01
Ascorbic acid	lower concentration	5.55	14.8
	higher concentration	0.65	-1.95

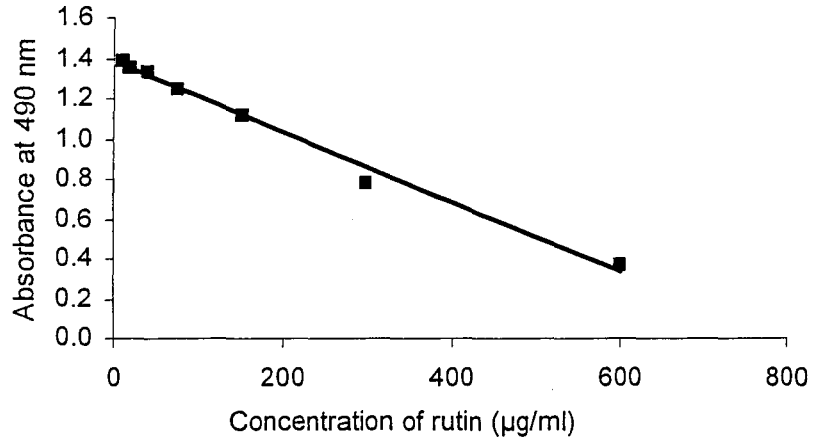


Figure 3.5 Calibration curve of rutin in the DPPH antioxidant assay (r^2= 0.994).

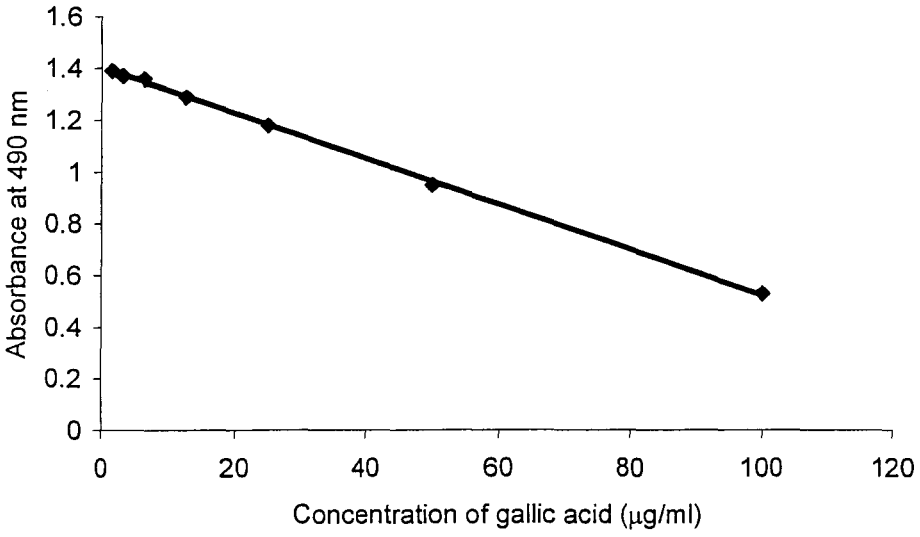


Figure 3.6 Calibration curve of gallic acid in the DPPH antioxidant assay (r^2= 0.999).

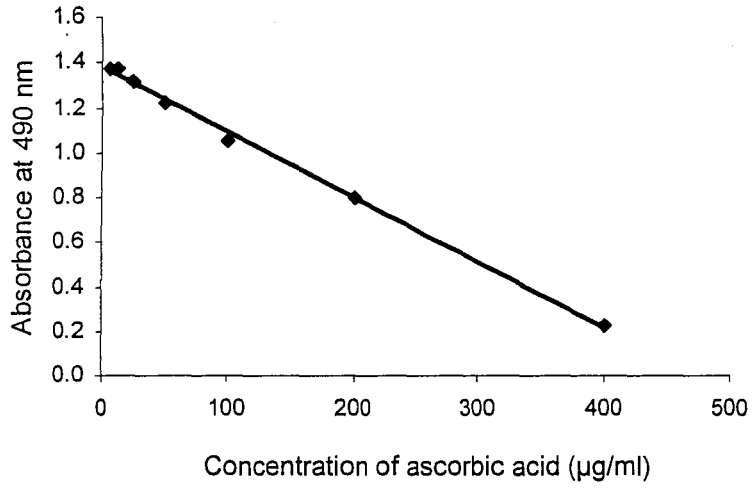


Figure 3.7 Calibration curve of ascorbic acid in the DPPH antioxidant assay (r^2= 0.998).

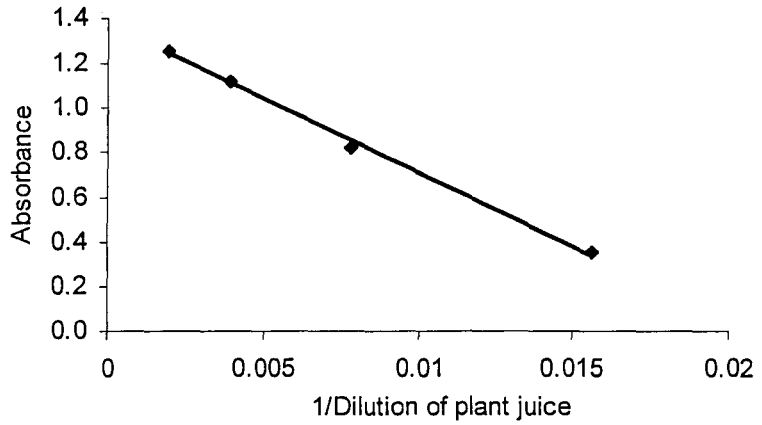


Figure 3.8 Plot of inverse of dilutions versus absorbance of *Carpobrotus edulis* (CeJ2) plant juice obtained in the DPPH antioxidant assay (r^2 = 0.996).

In the above figure the plant juice was prepared in dilutions of 4, 8, 16, 32, 64, 128, 256 and 512 times. There was excess antioxidant power present up to 32 times dilution. The absorbances of the excessively concentrated samples are not included in Figure 3.8.

Table 3.5 The DPPH assay EC_{50} values determined for each antioxidant standard under the described assay conditions.

Standards	DPPH EC_{50}
Ascorbic acid	203 μg/ml
Rutin	317 μg/ml
Gallic acid	65 μg/ml

Table 3.6 Antioxidant activity by the DPPH method of *Carpobrotus* species plant juices and the contribution of ascorbic acid to the antioxidant activity.

Plant juice codes	DPPH EC_{50} (dilutions)	DPPH EC_{50} rutin equivalent (mg/ml)	DPPH EC_{50} gallic acid equivalent (mg/ml)	DPPH EC_{50} ascorbic acid equivalent (mg/ml)	Plant juice Ascorbic acid concentration (mg/ml)	% DPPH activity due to ascorbic acid in plant juice	% DPPH activity due to antioxidants other than Ascorbic acid
Ce[a]J 1	116	37	7.6	24	1.76	7.5	93
CeJ 2	101	32	6.5	21	1.56	7.7	92
CeJ 3	15.5	4.9	1.0	3.2	0.49	16	85
Cr[b]J 1	55.3	18	3.6	11	1.02	9	91
CrJ 2	45.2	14	2.9	9.2	0.85	9.2	91
CrJ 3	44.5	14	2.9	9.0	0.59	6.6	93
Ca[c]J 1	31.7	10	2.1	6.5	1.08	17	83
CaJ 2	28.6	9.1	1.9	5.8	0.73	13	87
CaJ 3	52.1	17	3.4	11	0.86	8.1	92

[a]Ce is *C. edulis*, [b] Cr is *C. rossii*, [c] Ca is *C. aequilaterus*.

3.4.4 FRAP assay results

The antioxidant activity of *Carpobrotus* species plant juice using the FRAP assay are summarised in Table 3.9. The calibration curves of rutin, gallic acid and ascorbic acid are shown in Figures 3.10, 3.11 and 3.12. Calibration curve of ferrous sulphate is shown in Figure 3.9. A graph of inverse of dilution factor (concentration) versus FRAP value for CeJ2 is shown in Figure 3.13. The accuracy and precision of the FRAP assay was calculated and results are shown in Table 3.7.

Table 3.7 Accuracy and precision of FRAP assay.

FRAP assay		Precision %RSD (n=3)	Accuracy (%) (N=3)
Rutin	lower FRAP number	17.5	4.63
	higher FRAP number	0.68	< 0.01
Gallic acid	lower FRAP number	10.8	-0.28
	higher FRAP number	13.3	< 0.01
Ascorbic acid	lower FRAP number	8.31	-0.11
	higher FRAP number	19.1	-0.01
$FeSO_4$	lower FRAP number	3.93	0.14
	higher FRAP number	4.10	<-0.01

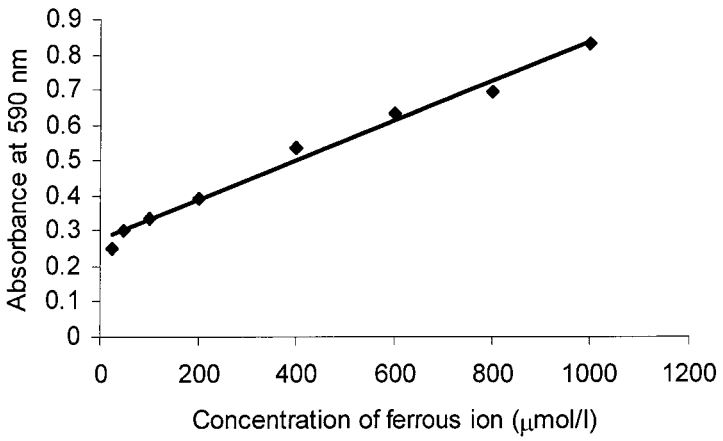


Figure 3.9 Calibration curve of ferrous sulphate for FRAP assay (r^2= 0.995).

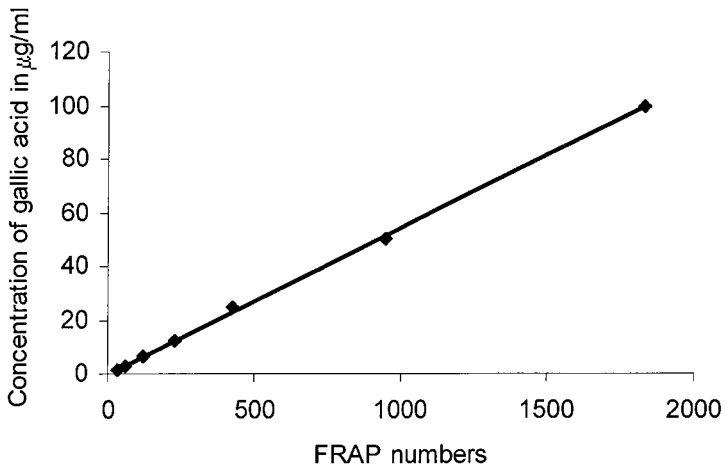


Figure 3.10 Plot of concentration versus FRAP value for gallic acid (r^2= 0.997).

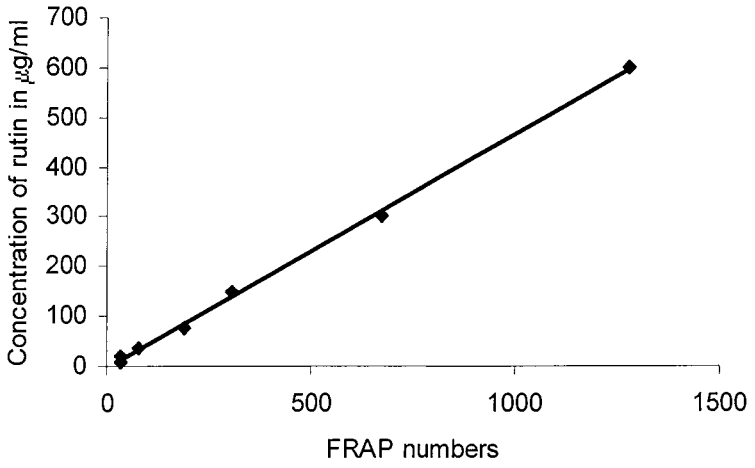


Figure 3.11 Plot of concentration versus FRAP value for rutin (r^2= 0.998).

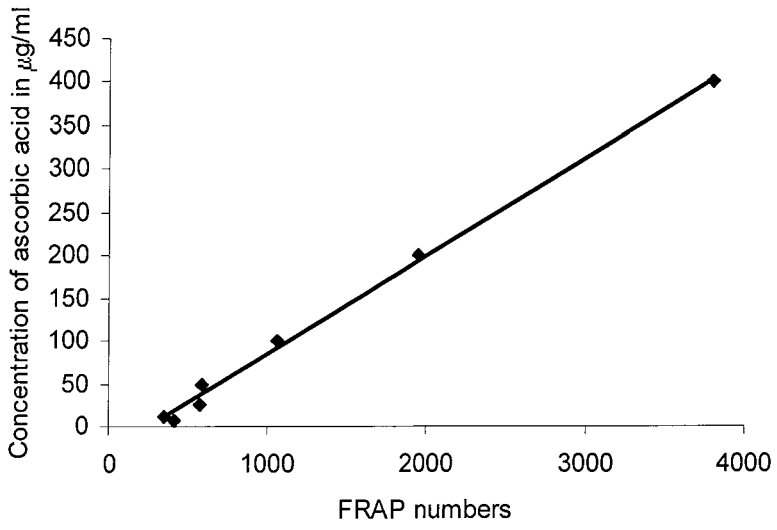


Figure 3.12 Plot of concentration versus FRAP value for ascorbic acid (r^2= 0.997).

Table 3.8 FRAP values of standards in the FRAP assay

Antioxidant standards	Concentration at 1000 FRAP value
Ascorbic acid	110 µg/ml
Rutin	350 µg/ml
Gallic acid	50 µg/ml

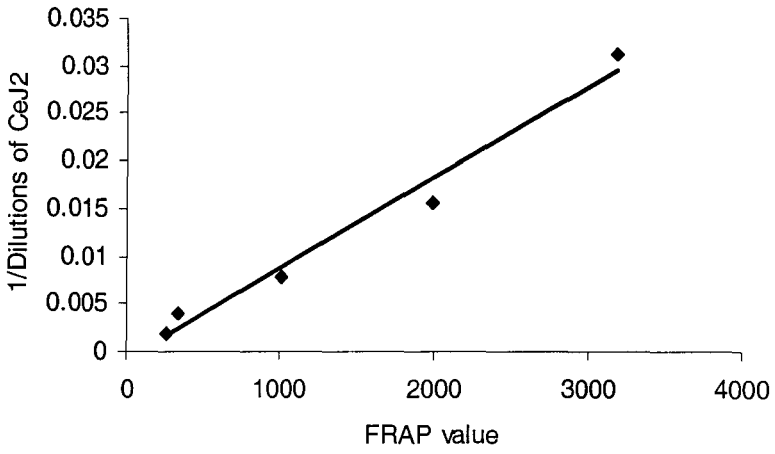


Figure 3.13 Plot of inverse of dilutions versus FRAP value for *C. edulis* (CeJ2) (r^2= 0.988).

Table 3.9 Different *carpobrotus* species plant juices antioxidant activity and the standards equivalents by FRAP assay, with ascorbic concentration in plant juices.

Plant juice codes	Dilution at FRAP value 1000	Rutin equivalent (mg/ml)	Gallic acid equivalent (mg/ml)	Ascorbic acid equivalent (mg/ml)	Plant juice Ascorbic acid concentration (mg/ml)	% FRAP activity due to Ascorbic acid in plant juice	% FRAP activity due to Antioxidants other then Ascorbic acid
Ce[a]J 1	106	38	5.4	11.8	1.76	14.9	85.1
CeJ 2	109	38	5.4	12	1.56	13	87
CeJ 3	15	5.3	0.8	1.7	0.49	28.7	71.3
Cr[b]J 1	118	41	5.9	12.9	1.02	7.9	92.1
CrJ 2	57	20	2.6	6.3	0.85	13.5	86.5
CrJ 3	35	12	1.8	3.9	0.59	15	85
Ca[c]J 1	56	19	2.8	6.1	1.08	17.6	82.4
CaJ 2	51	18	2.6	5.6	0.73	13.1	86.9
CaJ 3	116	41	5.8	12.8	0.86	6.7	93.3

[a]Ce is *C. edulis*, [b]Cr is *Carpobrotus rossii*, [c]Ca is *C. aequilaterus.*

3.4.5 Total phenolics assay results and discussion

The calibration curve of gallic acid is shown in Figure 3.14. The gallic acid equivalent of each plant juice was calculated by interpolation from the standard curve. The gallic acid equivalent values obtained were for the diluted plant juices. The total gallic acid equivalents were calculated by multiplying values by the dilution factor.

Total phenolics assay performance was assessed using the correlation coefficient of calibration curve of gallic acid, accuracy (observed – expected)/expected x 100 (n=3) and precision was reported as percent relative standard deviation (% RSD; n=3).

The results of total phenolics content of the *Carpobrotus* species plant juices are summarised in Table 3.10. The calibration curve for gallic acid is shown in Figure 3.14. The precision (% RSD) at the highest point was 0.53% (n=3) and at the lowest point was 5.45% (n=3). The accuracy at highest point was 0.08% and lowest point was 0.04%.

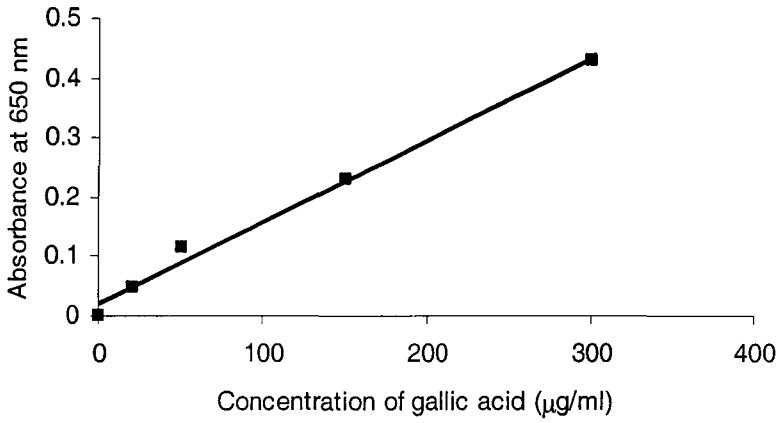


Figure 3.14 Calibration curve of gallic acid (r^2 = 0.989).

Table 3.10 Gallic acid equivalent (GAE) of different *Carpobrotus* plant juices.

Plant species	Absorbance	Gallic acid equivalent of diluted juice (GAE) (µg/ml)	GAE of plant juice (mg/ml)
Ce[a]J1	0.250	3260	3.26
CeJ2	0.220	2831	2.83
CeJ3	0.158	972	0.97
Cr[b]J1	0.381	2565	2.57
CrJ2	0.393	2651	2.65
CrJ3	0.294	1944	1.94
Ca[c]J1	0.158	972	0.97
CaJ2	0.388	2615	2.62
CaJ3	0.364	2444	2.44

[a]Ce is *C. edulis*, [b]Cr is *C. rossii*, [c]Ca is *C. aequilaterus*

Carpobrotus edulis from home garden (CeJ3) demonstrated considerably less phenolics compared with the other *C. edulis* plants, CeJ1 and CeJ2. *C. rossii* plants collected from the same locations were found to have a similar concentration of phenolics, whereas with *C. aequilaterus,* one of the plants had significant less phenolics compared with the other two plants. These variations may be due to differences in soil composition and other edaphic factors.

3.4.6 Analysis of antioxidants in *Carpobrotus rossii* plant juice using LC-MS

The LC-MS chromatogram of *C. rossii* plant juice antioxidant fraction is shown in Figure 3.15.

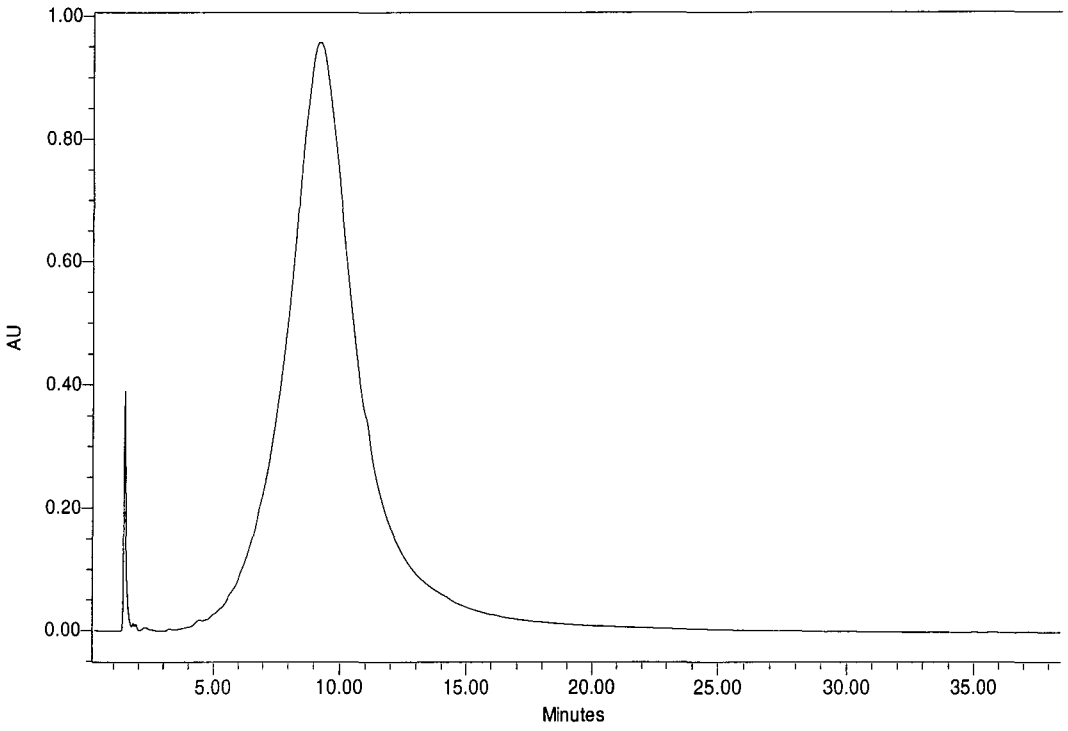


Figure 3.15 LC-MS chromatogram of *C. rossii* plant juice antioxidant fraction with UV detection at 280 nm.

Figure 3.15 shows that there was a broad peak which might have resulted from a mixed composition of many similar chemical constituents rather than several individual flavonoids. MS data from the peak (Figure 3.16) shows a large number of compounds or fragments with regular mass differences of 144 mass units, for example between peaks at *m/z* 720, 864, 1008, 1152, 1296 and 1440. These signals are consistent with condensed tannins that vary by 288 mass units, for example by addition of procyanidin units (Engelke *et al.*, 2004). The peaks at *m/z* 864 and 1152 are similar to those found in litchi extracts that were postulated as due to (epi)catechin trimers and tetramers (Sarni-Manchado *et al.*, 2000). The mass difference of 144 may represent acylation by the 3-hydroxy-3-methylglutaric acid moiety or similar, of some of the condensed tannins, as

postulated for the flavonoids in *C. rossii*. This evidence indicates that the *C. rossii* plant juice antioxidant fraction mainly consists of condensed tannins.

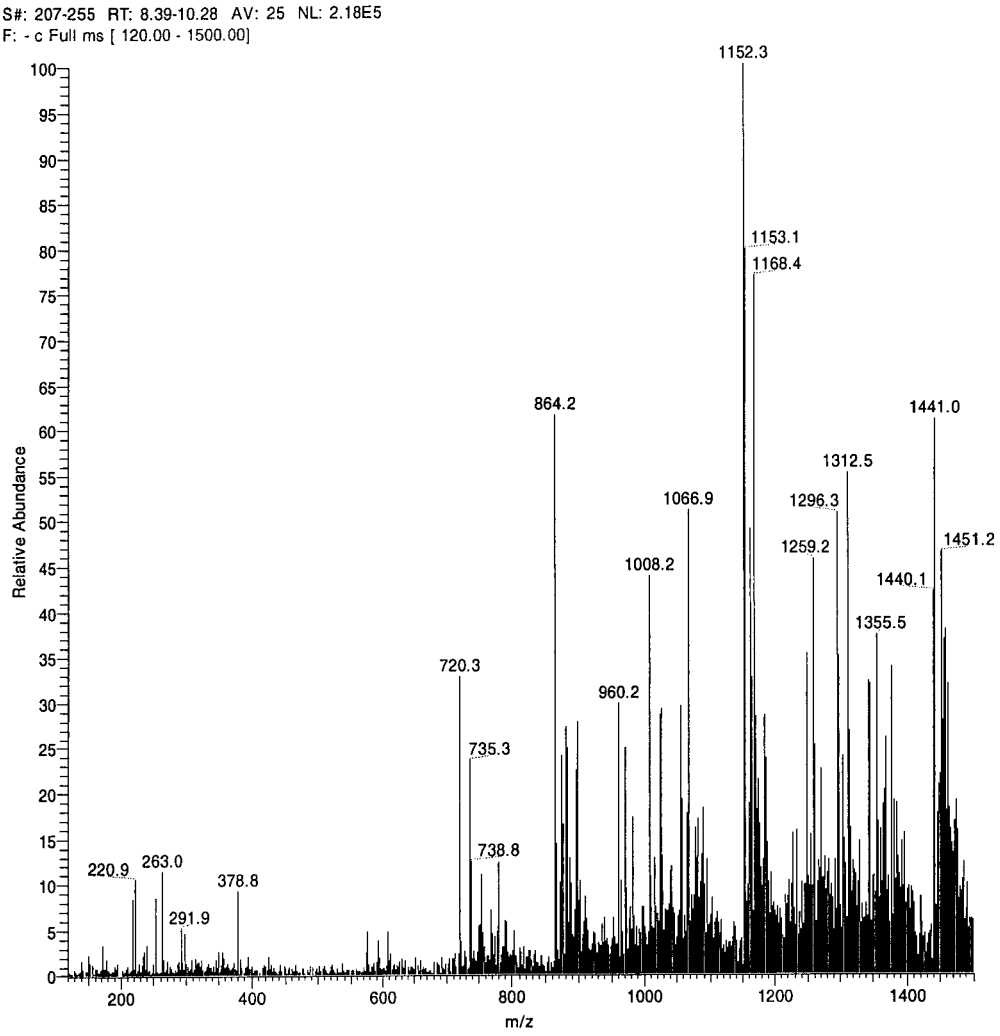


Figure 3.16 Mass spectrum of *C. rossii* plant juice antioxidant fraction.

3.5 Agreement between antioxidant assays

The results from the three different antioxidant assays were not well correlated. Among these, the FRAP assay results and the β-carotene assay results were better correlated than either one with the DPPH assay results. The correlation coefficient

between the DPPH and the FRAP assay results was 0.71. The β-Carotene assay results had a correlation coefficient with the DPPH assay results of 0.73 and with the FRAP assay results of 0.89. Gallic acid was the most potent antioxidant in every assay among the 3 standard antioxidants used. Ascorbic acid was the least sensitive standard antioxidant in the β-carotene assay and rutin was the least sensitive standard antioxidant in the FRAP assay.

The correlation coefficient between the DPPH and FRAP assays was only 0.71, when considering all samples of all species of *Carpobrotus*, but it was good between samples from the same species. The correlation coefficient between the two assays for *C. edulis* samples was 0.98, for *C. rossii* was 0.97 and for *C. aequilaterus* was 0.99.

3.6 Discussion

Antioxidants are a heterogeneous group of chemicals that have the ability to slow or block oxidation processes and prevent free radical-dependent damage occurring to cellular constituents such as proteins, nucleotides, phospholipids and polyunsaturated fats. The use of antioxidants as a primary defence mechanism to combat the damaging effects of free radicals makes them an interesting family of molecules for applications in nutrition and cosmetics (Buenger *et al.*, 2006).

Antioxidant properties in plants are usually possessed by polyphenolics, ascorbic acid, carotenes and other vitamins. So the presence of flavonoids and polyphenolics in *Carpobrotus* species plants indicated the presence of potential antioxidants. Many

analytical methods have been used to measure the antioxidative activity of individual chemical substances or of plant juices and extracts.

In this study, three assays were used to assess the antioxidant potential of *Carpobrotus* species plant juices, namely the DPPH assay, the FRAP assay and the β-carotene bleaching method. Each of the methods has its advantages and disadvantages, with different mechanisms of action which should be considered when analysing the results. The DPPH assay was easy to perform with little time required to prepare reagents and ample time to read results, with an incubation period of 45 minutes. The FRAP assay was also considerably easy with regard to reagent preparation, but the timing to read the microtitre plate results was more critical. The required incubation period was only 8 minutes. Given the time required to pipette out the reagents into the microtitre plate using a single transfer pipetter would typically be more than one minute, incubating all wells in the plate for the same 8 minute period was difficult. The β-carotene bleaching method was a more tedious method, involving preparation of an emulsion and an incubation period of 2 hours.

From the literature it has been found that among the three antioxidant assays, the DPPH assay yields the best results based on the sensitivity and reproducibility within inter-laboratory and intra-laboratory determinations. Results of the assays also depend on the training, equipment and the scale of measurement (Buenger *et al.*, 2006).

The antioxidant assays were modified in order to perform them on a microtitre plate. This made them easier assays to perform and used lesser amounts of reagents. The time taken to perform the assays was also decreased and samples could be routinely

analysed in triplicate, which gave consistency in results. There are other new techniques for the assessment of antioxidant activity using the same reagents. A novel electrochemical method for the selective detection of antioxidant based on the 2,2-diphenyl-1-picrylhydrazyl/2,2-diphenyl-1-picrylhydrazine redox couple and employing a biamperometric technique was developed. This redox couple exhibited a high degree of reversibility (Milardovic *et al.*, 2005). These newer techniques may help in prevention of human errors and errors due to scale of measurements.

In all the antioxidant assays, three common antioxidant standards were used: gallic acid, rutin and ascorbic acid. Among these standards gallic acid proved to be the most potent in all three assays. Ascorbic acid was the second most potent in the DPPH and FRAP assays but with the β-carotene bleaching assay it was the least potent. The lower sensitivity of ascorbic acid in the β-carotene assay may be due to the lipophilic system of the assay. Ascorbic acid is very hydrophilic in solution (Castelluccio *et al.*, 1996). The potency of rutin was opposite to the findings for ascorbic acid. It was the least potent in the DPPH assay and the FRAP assay while second most potent in the β-carotene assay. This might be due to rutin, like ferulic acid, being more lipophilic than ascorbic acid (Castelluccio *et al.*, 1996). These results are consistent with the literature, as it is reported that gallic acid is a stronger antioxidant than rutin and ascorbic acid when tested with the DPPH and the FRAP assays (Schlesier *et al.*, 2002).

The DPPH assay results were analysed by determing DPPH EC_{50}, the concentration at which 50% of the initial concentration of DPPH is reduced. The concentration of rutin to achieve DPPH EC_{50} was more than that reported in other studies. This higher rutin

concentration was possibly due to a doubling of the initial concentration of DPPH used in this experiment compared with the conventional method. The concentration of DPPH was doubled because for a microtitre plate, with shorter path length than a typical cuvette, a higher concentration was required to achieve a similar initial absorbance (> 1).

The antioxidant potential of *Carpobrotus* species plant juice was very high compared with other known naturally occurring antioxidants like tea, orange juice and red wine. CeJ2 plant juice was taken as the example and its FRAP value was compared with the FRAP value of other naturally occurring antioxidants. CeJ2 had a FRAP value of 109 mmol/L of Fe^{2+} ion concentration. Table 3.11 gives a range of naturally occurring vegetables, fruits and beverages with their FRAP values (Pellegrini *et al.*, 2003). It also shows their equivalent multiplying factor to achieve that of CeJ2 antioxidant power. The antioxidant potential of CeJ2 juice is equal to twice that of blackberries and the same as coffee (soluble).

Table 3.11 FRAP values of different natural products[a] and a comparison with *Carpobrotus edulis* juice CeJ2.

Vegetable	FRAP value mmol Fe^{2+}/kg	% CeJ2 activity	Fruits	FRAP value mmol Fe^{2+}/kg	% CeJ2 activity	Beverages	FRAP value mmol Fe^{2+}/L	% CeJ2 activity
carrot	1.1	1.0	apple	3.8	3.5	tea (green)	18	16.5
potato	3.7	3.4	kiwi fruit	7.4	6.8	tea (black)	10.1	9.3
beetroot	15.3	14.0	strawberry	28.0	25.7	wine (red)	23.9	21.9
broccoli	11.7	10.7	raspberry	43.0	39.4	wine (white)	3.72	3.4
spinach	26.9	24.7	blackberry	51.5	47.2	coffee (soluble)	108.6	99.6
mushroom	16.4	15.0	redcurrant	44.9	41.2	orange juice	9.44	8.7

[a] from Pellegrini *et al.*, 2003.

The FRAP values given by Pellegrini *et al.,* (2003) take no account of ascorbic acid levels in the natural products and the contribution of ascorbic acid antioxidant activity.

The contribution of ascorbic acid to the antioxidant properties of *Carpobrotus* species plant juice is not a major part of the activity in most cases and typically accounts for only about 10% of the total antioxidant activity. The ascorbic acid concentration in the *Carpobrotus* species plant juices is however, considerable compared to its concentration in other succulents. The cactus, *Opuntia* species has an ascorbic acid content of 18-23 mg/100 in its pears (Stintzing *et al.*, 2001). *Carpobrotus* species juice had ascorbic acid concentrations ranging from 49-176 (mean 100) mg/100 ml, 5 times greater than levels in the cactus. Orange juice has an ascorbic acid concentration of 50 mg/100 ml and pineapple juice has 10-15 mg/100 ml (Alamo *et al.*, 1993). *Carpobrotus* was used for the treatment and prevention of scurvy by early Europeans.

From the analysis of the *C. rossii* plant juice antioxidant fraction by LC-MS it was found that the fraction consisted mainly of condensed tannins and polyphenolics. This fraction was the only fraction collected from the Sephadex LHLQ column that showed significant antioxidant activity by the DPPH assay. This indicates that the main cause of antioxidant activity in *C. rossii* is polyphenolics. These typically consist of catechins, flavonoids and tannins (condensed tannins). The total phenolics present in *Carpobrotus* species juice were assayed by using Folin-Ciocalteau reagent and the results were expressed in gallic acid equivalents. The levels of total phenolics present in *Carpobrotus* species juice were in the range of 0.97 – 3.26 (mean 2.25) mg/ml gallic acid equivalents (GAE). Phenolic content expressed as the equivalent

amount of gallic acid obtained by this assay was less than the gallic acid equivalents from the DPPH antioxidant assay: 1.0-7.6 (mean 4.3) mg/ml GAE, or FRAP assay: 0.8-5.9 (mean 3.35) mg/ml GAE. Gallic acid is a part of the polyphenolics system and there may be additional antioxidants, higher in potency than gallic acid, such as catechin, epicatechin, condensed tannins and other flavonoids, as well as non-phenolics antioxidants, that contribute to the total antioxidant properties.

Cactaceae members, which are succulents, typically have low levels of polyphenols (0.39 mg/g) compared with *Carpobrotus* (Stintzing *et al.*, 2001). The antioxidant activities of these plants also are very low when compared with *Carpobrotus* species, consistent with the contribution of polyphenols to the antioxidant activity of *Carpobrotus* species.

The amount of total phenolics assayed by the Folin-Ciocalteau method in black tea was 1.36 mg/ml, in red wine was 0.77–3.2 mg/ml and in black currant juice was 1.15–1.76 mg/ml. (Henn and Stehle, 1998). Tea contains polyphenols such as catechin and flavan-3-ols including epicatechin and epicatechin gallate (Seeram *et al.*, 2006). Polyphenols account for the major antioxidant activity of tea. These beverages are considered to be rich source of polyphenolic substances, therefore *Carpobrotus* species juices can also be considered rich in polyphenolics.

There appeared to be some variations in the antioxidant potency between species as well as within species of *Carpobrotus*. All three plants from each species of *C. rossii* and *C. aequilaterus* had similar antioxidant activities with CrJ1 and CaJ3 slightly higher. Among the three species *C. edulis* demostrated a higher antioxidant potential

by all three antioxidant assays. Two plants of *C. edulis*, CeJ1 (5.4 mg/ml GAE by FRAP assay, 7.6 mg/ml GAE by DPPH assay and 2.5 mg/ml GAE by β-carotene assay) and CeJ2 (5.4 mg/ml GAE by FRAP assay, 6.5 mg/ml GAE by DPPH assay and 2.0 mg/ml GAE by β-carotene assay) were found to have a very high antioxidant potential whereas CeJ3 (0.8 mg/ml GAE by FRAP assay, 1.0 mg/ml GAE by DPPH assay and 0.3 mg/ml GAE by β-carotene assay) had the lowest antioxidant activity among all the species. It was also noted that CeJ3 had lower levels of sodium when compared to levels of potassium, which was very different to levels of these ions in the other eight plants of *Carpobrotus* species.

There was also a definite difference in plant chemical constituents such as total phenolics in CeJ3, which may be the reason for the lower antioxidant activity. The hydration levels of the plant material may also effect the chemical constituent concentration and therefore the antioxidant activity of the plant juice. The plant that gave CeJ3 juice was grown in a well-watered, fertilized garden plot, compared with the other examples of *C. edulis* which were grown by the road side. These results suggest that edaphic factors at the growing site may strongly influence the chemical composition of *Carpobrotus* species plants and their properties. For example a study was conducted on the effect of different concentrations of mineral nitrogen in the soil on the growth and chemical constituents of carrot. The study showed that there was a linear increase in total sum β-Carotene with increase in mineral nitrogen in the soil (Kaack *et al.*, 2001).

There are other natural products which are used either used as medicines or herbal supplements for their antioxidant properties. Brazilian research showed that a local

plant extract cured peptic ulcers caused by excessive consumption of ethanol. The anti-ulcerogenic pharmacological effect of these plants was related to their flavonoid content and antioxidant properties (Repetto and Llesuy, 2002). Tebonin is an over-the-counter drug introduced into the Australian market for its use to relieve the symptoms of tinnitus. The preparation contains *Gingko biloba* extract, EGb761, which has potent antioxidant properties believed to improve circulation and protect adenosine triphosphate (ATP) (DeFeudis and Drieu, 2000).

The therapeutic potential of *Carpobrotus* species is significant given the high antioxidant activity of the plant juice. Further research is strongly recommended for exploring the dietary and therapeutic potential of *Carpobrotus* species juices.

Chapter 4: Clinical trials

4.1 Histamine skin-prick antiinflammatory model

4.1.1 Objective

To test the antiinflammatory action of *Carpobrotus rossii* plant juice extracts against histamine responses in humans using a double-blinded placebo-controlled skin prick test.

4.1.2 Introduction

The skin prick test is a fundamental test for the evaluation of changes in skin sensitivity due to treatment and in biological allergen standardisation (Zimmerman, 1998). Histamine is the basic amine present in the body which is released during injury and is a part of the cascade of reactions that cause inflammation (Rang *et al.*, 2003). Histamine is known to cause the "triple response" of Lewis. In humans, the dermal triple response to histamine consists of an initial redness due to direct vasodilation, a subsequent irregular brighter flush (flare) due to axon reflexes, and a wheal due to increased capillary permeability. Histamine acts via H_1 and H_2 receptors. Inflamed tissues have elevated levels of histamine. Mast cells produce and store histamine and the mechanism for histamine release involves mast cell surface receptors.

There are anecdotal reports that *Carpobrotus* plant leaf juice is effective in soothing itching caused by spider and tick bites (Roberts, 1990). The juice is highly astringent

and used externally on wounds, burns and to treat eczema. *Carpobrotus* juice also has been used to treat bluebottle, jellyfish sting and burns. This study investigated the potential antiinflammatory activities of *Carpobrotus* plant juice, acting through inhibition of histamine-mediated processes.

4.1.3 Materials

Materials used for this study included: histamine dihydrochloride, chlorohexidine gluconate (Sigma-Aldrich, Sydney, Australia); gum tragacanth powder (D. Craig BP, repack); vanillin (Sigma Chemical Company, USA); glycerol (Natural Oleo Chemicals, Sydney, Australia) and 5 ml screw cap aluminium ointment tubes (G.E. Crane and Sons, Victoria, Australia).

4.1.4 Preparation of histamine solution

Histamine is available in salt form as the dihydrochloride and the acid phosphate. A mass of 1.66 g of histamine dihydrochloride is equivalent to 1 g of histamine. The histamine solution was prepared by dissolving 41.5 mg of histamine dihydrochloride in 25 ml of pure distilled freshly boiled and cooled water to obtain a 1 mg/ml histamine solution. The histamine solution was then dispensed into 1 ml amber coloured glass ampoules which were flame sealed. The ampoules were sterilised using an autoclave (121 $^{\circ}$C for 15 mins).

4.1.5 Method

4.1.5.1 Freeze drying of the plant juice

Carpobrotus rossii was collected from Seven Mile Beach, in Southern Tasmania, 42° 50′ S, 147° 32′ E. Voucher specimens were lodged with the Tasmanian Herbarium, Hobart, Tasmania for preservation and identification with voucher numbers for the plants: HO 529461 (*C. rossii*), and HO 529462 (*C. rossii*).

C. rossii plant leaf was macerated using a blender to obtain 1 litre of plant juice. Small particles from the macerated plant material were allowed to settle at room temperature for 30 minutes and then filtered using vacuum through Whatman filter paper (no. 10) to obtain clear plant juice. Aliquots of the plant juice were then packed in six zip-lock seal plastic bags and frozen at -18 °C. The ice blocks of plant juice were then removed from the plastic bags and placed in trays of a freeze dryer. The tray surfaces were covered in aluminum foil to prevent contamination from the tray surface. The frozen plant juice was freeze dried for 3 days. The dried plant extract yielded from the aluminum foil 33 g of sticky pink solid which was packed in a zip-lock sealed plastic bag and stored in a freezer at -18 °C. Some of the extract could not be removed from the aluminum foil

4.1.5.2 Preparation of gel

Carpobrotus rossii plant juice was formulated as a topical gel and prepared in the School of Pharmacy laboratory at the University of Tasmania. The formulation was based on Chlorohexidine Gel APF 17th Edition (Australian Pharmaceutical Formulary

and Handbook) and included the dried *C. rossii* plant juice extract in the test formulation. The standard Chlorohexidine Gel APF, without *C. rossii* plant juice was prepared as the control formulation. In order to assist in blinding investigators and study subjects, 1% of vanillin was added to both test and control gels to mask the subtle odour of the *C. rossii* plant juice extract in the test gel.

The gels were prepared according to the following procedure:

Chlorohexidine Gel APF 17th Edition

Chlorohexidine gluconate	2.5 ml
Tragacanth powder	2.5 g
Glycerol	25 ml
Purified water, freshly boiled and cooled	to 100 g (about 70 ml)

1. The gel was made in a clean area within the dispensing laboratory.
2. Tragacanth powder (2.5 g) was weighed in to a clean, dry, tared glass beaker.
3. Glycerol (25 ml) was added to the tragacanth powder in the beaker and was mixed very well with the glycerol using a glass stirring rod. (Tragacanth forms lumps on contact with water unless dispensed well in an agent such as glycerol).
4. Purified water was freshly boiled and cooled (70 ml). Approximately 60 ml was added to the paste of tragacanth and glycerol and mixed well using a glass stirring rod.
5. The gel was heated until boiling by placing the beaker directly on a hot plate, and then stirred frequently until the first bubble appeared. The gel was than removed from the hot-plate.

6. When the gel was cooled, 2.5 ml of chlorohexidine gluconate solution BP were added.
7. Dried *C. rossii* plant juice extract (3.35 g) was dissolved in water (approximately 5 ml) and combined with the gel.
8. Vanillin (1 g) was dissolved in the last part of water (5 ml) and added to mask the subtle *C. rossii* odour.

The control gel was prepared by the above procedure except for the addition of dried *C. rossii* plant juice extract. The gels were packed in 5 g screw cap ointment aluminium tubes. The gel was stored at room temperature with an expiry of 28 days.

The concentration of solids in *C. rossii* plant juice extract was 33.5 mg/ml, a concentration determined by the evaporation of 1 ml of fresh plant juice. The gel was formulated to contain the same concentration of *C. rossii* solids as the plant juice in order to simulate the chemical properties of the natural plant juice in the gel preparation as closely as possible.

4.1.5.3 Stability of the plant juice extracts gel

4.1.5.3.1 Stability test using HPLC

The stability of the gel was investigated after autoclaving and boiling. The gel, which was packed in 5 g aluminium tubes, was subjected to autoclaving at 121 °C for 15 minutes. Another tube of gel was placed in a beaker of water and boiled at 100 °C for 60 minutes. The autoclaved gel, boiled gel and control gel (gel not subjected to heat

stress) were then assessed by HPLC to detect any noticeable changes in constituents of the gel.

The gel and reconstituted plant juice extract were also analysed for flavonoid content by using HPLC. Gel was diluted 10-fold with water, then sonicated for 15 minutes. The aqueous solution was subjected to solid phase extraction using a Phenomenex Strata-X cartridge® and eluted with 1 ml of 80% methanol/water. *C. rossii* plant juice extract was reconstituted with water to the same strength as the original plant juice, diluted 10-fold with water and extracted in the same way as the gel.

The HPLC system consisted of a Varian Prostar 230 solvent delivery module, Prostar 330 photodiode array detector and Prostar 440 auto sampler. An injection volume of 10 μl was used with UV detection at 280 nm. The gel was dissolved in water for injection of the sample. The chromatography utilised a Waters Nova-Pak® C18 (150 mm x 3.9 mm) column and ODS guard column with a flow rate of 0.8 ml/min. A linear gradient elution profile was followed from 80% A (water with 2% acetic acid) and 20% B (methanol with 2% acetic acid) to 100% B over 35 minutes.

4.1.5.3.2 Plant juice extract gels microbial contamination

It was attempted to pass the gel through filters to reduce the load of any microbial contamination. Natural products typically have high levels of microbial contamination. Filters used were a 0.45 μm CHROMACOL®, 30 mm, nylon filter with a 1 μm pre-filter. Gel was taken in a syringe and then tip of the syringe was attached to the pre-filter using the Luer-lok® which was than connected to the

0.45 μm filter. Pressure was applied on the syringe to pass the gel through both the filters in series.

4.1.5.4 Study protocol

Participants were human subjects recruited from staff and students at the University of Tasmania. There were notices and posters displayed around the university campus to notify the potential participants about the study. Bachelor of Pharmacy students were also made aware of the study. Subjects who were healthy and had no eczema or infection on their forearm were eligible for the investigation. Exclusion criteria for subjects (Volcheck, 2001) are shown in Table 4.1.

Table 4.1 Exclusion criteria for potential subjects for the histamine skin prick test

Subjects below the age of 18 years.
Skin infections.
Inflamed forearm.
Forearm with eczema.
Immunosuppressed subjects.
People with other skin diseases.
Subjects on other anti-inflammatory medication or therapy.
Pregnant and lactating women.
History of food and serious allergies or allergic responses.
Subjects on first-generation anti-histamines, topical corticosteroids, benzodiazepines, oral corticosteroids and non-sedating anti-histamines[5]

4.1.5.5 Study design

The investigation was placebo controlled and double-blinded. Subjects acted as their own control with the placebo and *Carpobrotus* gels tested on each subject on different arms. Both the subject and investigator were blinded. The randomisation was done by a person who was otherwise not involved in the study. Gels, test and placebo, were given a consecutive numerical subject code, and one gel was assigned for the left arm and the other for the right arm on the basis of the randomisation schedule, which was determined by a computer-generated list of random numbers. The randomisation schedule was released at the completion of all the experimental evaluations. The outcome measures were the area of flare and wheal, determined 15 minutes after the skin prick as well as the itch response. Two-tailed paired t-test was used for comparison between test and control formulations in each subject with $p<0.05$ considered statistically significant. The Chi-square test was used to assess the presence of blood at the prick site. Statistical analyses were performed using Statview for Macintosh version 5 (SAS Institute, Sydney). Twelve subjects were recruited. This study was approved by the Sothern Tasmanian Health and Medical Human Research Ethics Committee.

4.1.6 Skin – prick experimental method

On enrolling, subject's personal data (sex, age and name) and brief case history (skin allergies, other anti-inflammatory medications) were recorded. Subjects washed their hands and forearms with soap and cold water. Both forearm anterior skin surface were sterilized with ethanol swabs. One gel, containing either placebo or *Carpobrotus* juice extract, was applied by the investigator to one forearm and the

other gel was then applied to the other forearm. Approximately 2 ml of gel were used in each application. The gels were allowed to dry on the forearms (approximately 5 minutes). One drop of sterile histamine solution (prepared as described in 4.1.4) was placed on each of three different sites on the anterior forearm. The skin was pricked through each drop with a sterile 26 G needle (McLean, 1996). Each subject was pricked once with the needle at each site on the forearm and the needle entered only the dermis. The primary investigator performed each prick to reduce variability and errors. Thirty seconds after the skin prick the excess histamine solution was removed by swabbing with a tissue paper. The same process was then applied to the subject's other forearm.

After 15 minutes the intensity of itching of the first-treated forearm was noted on a scale of 0-10 with 10 "the most intense itching" and 0 "no itching". The areas of wheal and flare developed after 15 minutes were recorded by tracing their margins, using a felt tip pen directly onto the skin. The recordings were transferred by sticking clear adhesive tape to the tracing on the skin and removing the tape, which was left with an ink impression from the skin. The same process was then applied to the other forearm. The recordings were then photocopied and the areas of wheal and flare determined by the cut-and-weigh method, using the paper photocopied images.

4.1.7 Results

4.1.7.2 Stability of the plant juice extracts gel

HPLC analysis of the gel containing *C. rossii* plant juice was performed as a part of the stability study. The HPLC chromatograms (n=1) of the gel after autoclaving, after 2 months at room temperature and freshly prepared gel are shown in Figures 4.1, 4.2 and 4.3.

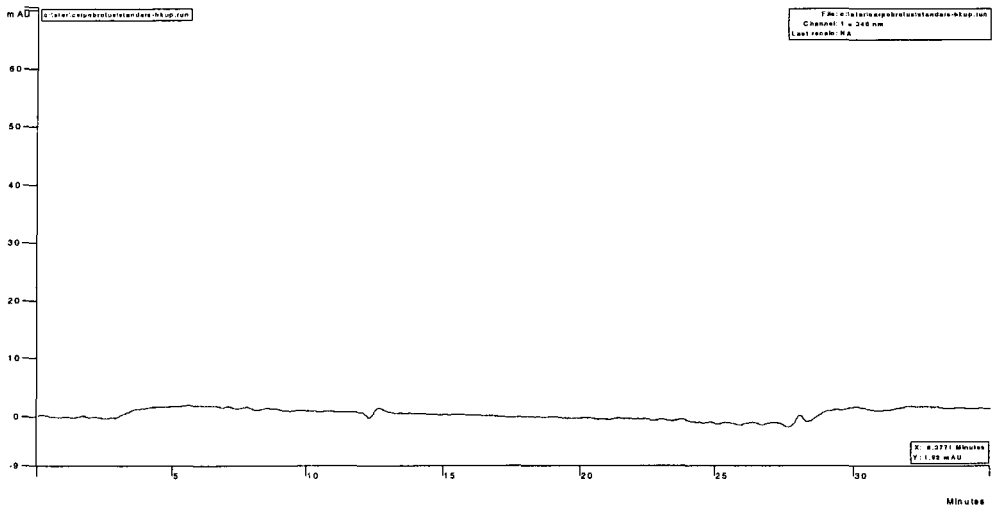


Figure 4.1 HPLC chromatogram of freshly prepared gel containing *C. rossii* plant juice.

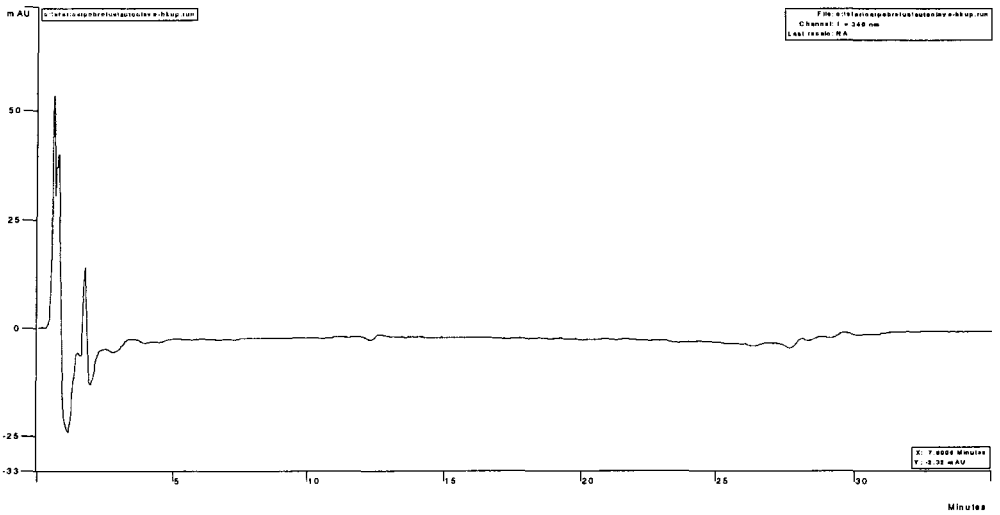


Figure 4.2 HPLC chromatogram of autoclaved gel containing *C. rossii* plant juice

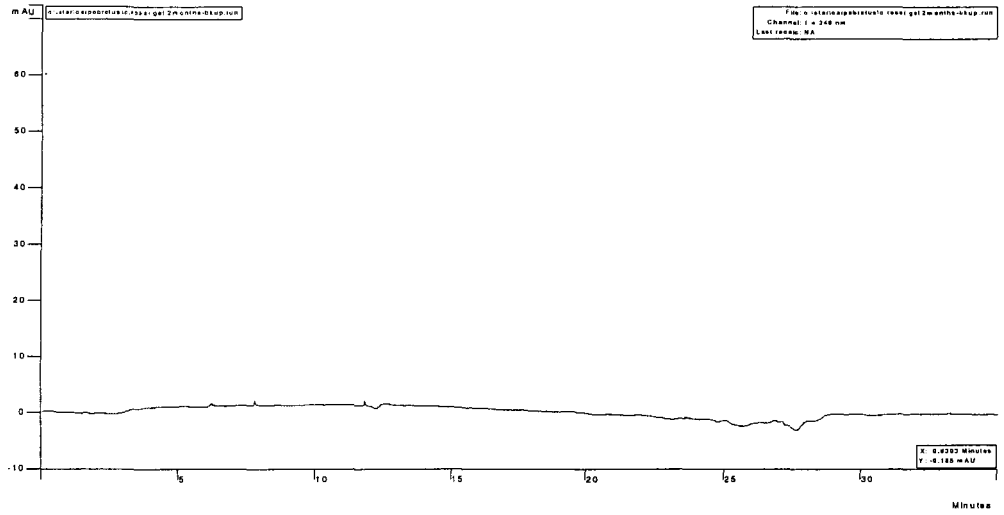


Figure 4.3 HPLC chromatogram of 2 months old gel containing *C. rossii* plant juice

From the HPLC chromatograms it can be seen that there was little change in the chemical constituents of the gel after storage for 2 months. But there was obvious change in the gel after undergoing the autoclaving process, with several new peaks evident in the chromatogram at retention times less than four minutes. The absence of the flavonoid peaks in the gels may be due to the trapping of flavonoids by gel matrix.

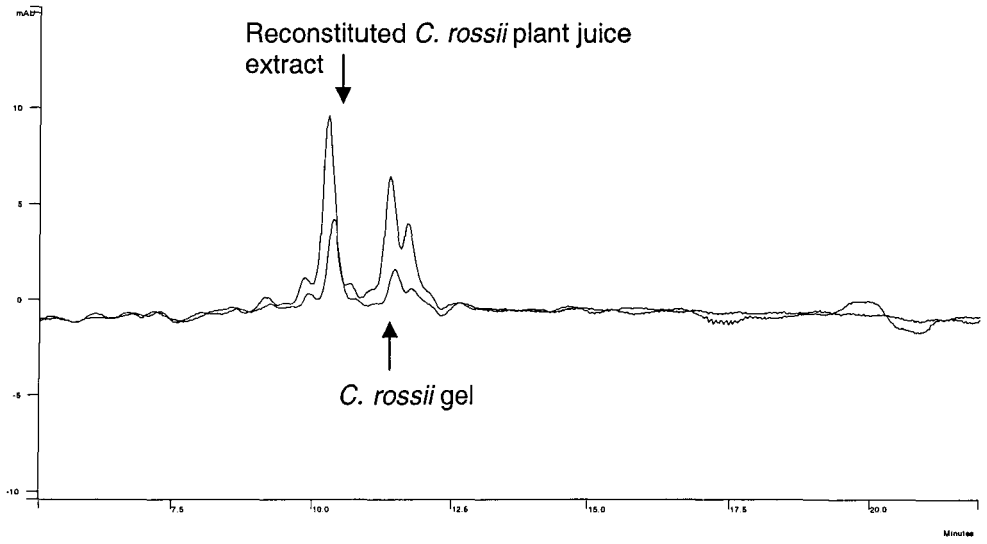


Figure 4.4 HPLC chromatogram of gel and reconstituted *C. rossii* plant juice after solid phase extraction.

From the HPLC chromatogram (Figure 4.4) the flavonoid peaks typically present in the *C. rossii* plant juice can be seen. The gel and the reconstituted juice both show the presence of the flavonoid peaks, but the reconstituted juice showed higher concentrations of the flavonoids compared with the gel. This may be due to trapping of some flavonoids by the gel matrix as evident in the earlier chromatograms. This experiment showed that the flavonoids were still present in the gel and not destroyed during the preparation of the gel.

4.1.7.3 Plant juice extract gels microbial contamination

Filtration was considered as an alternative method to autoclaving for the sterilisation of the gels. However the gel was not able to pass through the filters due to its viscosity even when heated.

4.1.7.4 Histamine skin prick test

The typical examples of the triple response in subjects following the histamine prick test are illustrated in Figure 4.4. The results of area of flare and wheal, itching, and the presence of blood on prickings in the control and test arms following the histamine skin prick test in 12 subjects are shown in Table 4.2. For the test *Carpobrotus* gel, flare area was 2.56±2.56 cm^2 (mean±SD), wheal area was 0.19±0.07 cm^2 (mean±SD) and itching score was 4.2±1.6 (mean±SD). Similarly for placebo gel, flare area was 2.75±1.54 cm^2 (mean±SD), wheal area was 0.19±0.05 cm^2 (mean±SD) and itching score was 3.5±2.0 (mean±SD). Figures 4.5, 4.6 and 4.7 show the itching score, wheal area and flare area following the histamine skin prick test after application of placebo and *C. rossii* gels.

A paired t-test comparison between active and control formulations showed no difference for either flare (df=11, t=-0.438, p=0.67), wheal (df=11, t=-0.426, p=0.68) or itching score (df=11, t=1.036, p=0.32). There was no difference between test and control for the observation of blood at the injection site (χ^2=6.0, df =1, Fisher's exact p=0.061) with 6/12 bleeding in the control group compared with 4/12 in the *carpobrotus* gel group.

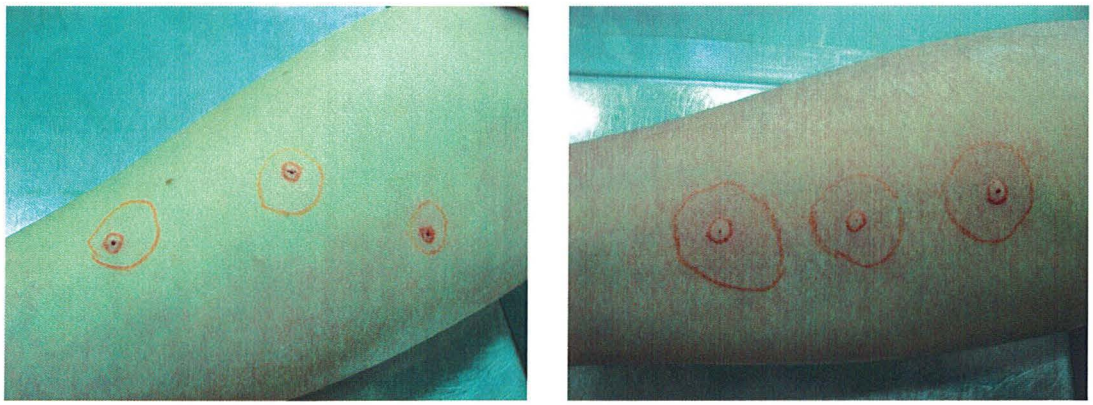


Figure 4.5 Triple responses in subjects following histamine prick test.

Table 4.2 The area of wheal and flare, itching response and the presence of blood on prick, on control and test arms in the histamine prick test.

Subject	Age	Gender	Area of flare in treatment arm (cm^2)	Area of flare in control arm (cm^2)	Area of wheal in treatment arm (cm^2)	Area of wheal in control arm (cm^2)	Itching scale in treatment arm[1]	Itching scale in control arm	Blood on prick in treatment arm[2]	Blood on prick in control arm
1	43	M	7.95	6.02	0.36	0.21	6	1	0 (no)	1
2	48	M	0	3.73	0.2	0.2	6	8	1 (yes)	0
3	27	M	6.59	4.77	0.17	0.22	1	1	0	1
4	51	F	1.15	1.37	0.24	0.24	5	4	1	0
5	21	F	1.62	1.73	0.14	0.21	5	3	0	1
6	21	F	0.96	2.04	0.11	0.15	6	3	0	1
7	21	F	4.67	3.9	0.15	0.11	4	3	1	0
8	50	F	3.32	3	0.17	0.1	3	6	0	0
9	20	M	1.39	1.34	0.19	0.26	5	3	0	1
10	21	M	1.65	1.64	0.22	0.24	3	4	0	1
11	25	F	0	1.41	0.16	0.22	3	2	0	0
12	20	M	1.46	2.08	0.11	0.16	3	4	1	0
Mean±SD	30.67		2.56±2.56	2.75±1.74	0.19±0.07	0.19±0.05	4.17±1.59	3.50±1.98	0.33±0.49	0.50±0.52

[1]Assessed by subjects on a scale of 0-10, [2] bleeding signified by 1 and no bleeding signified by 0.

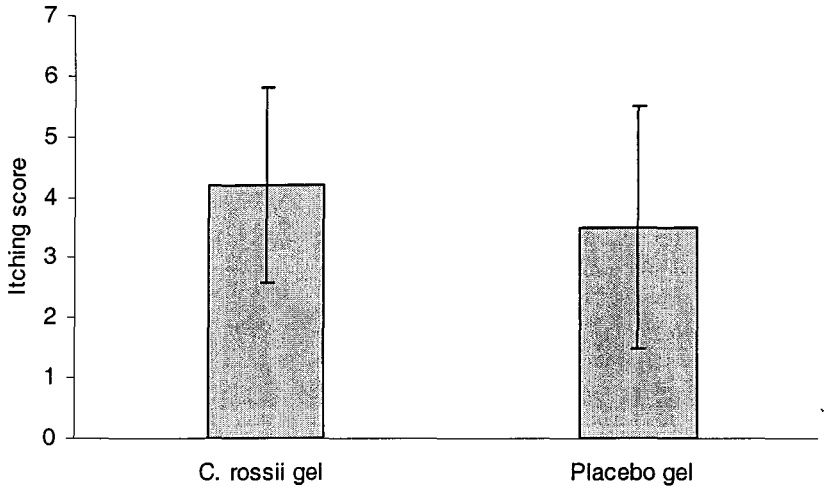


Figure 4.6 Itching responses to the histamine prick test (mean ± SD) after application of *C. rossii* gel or placebo gel.

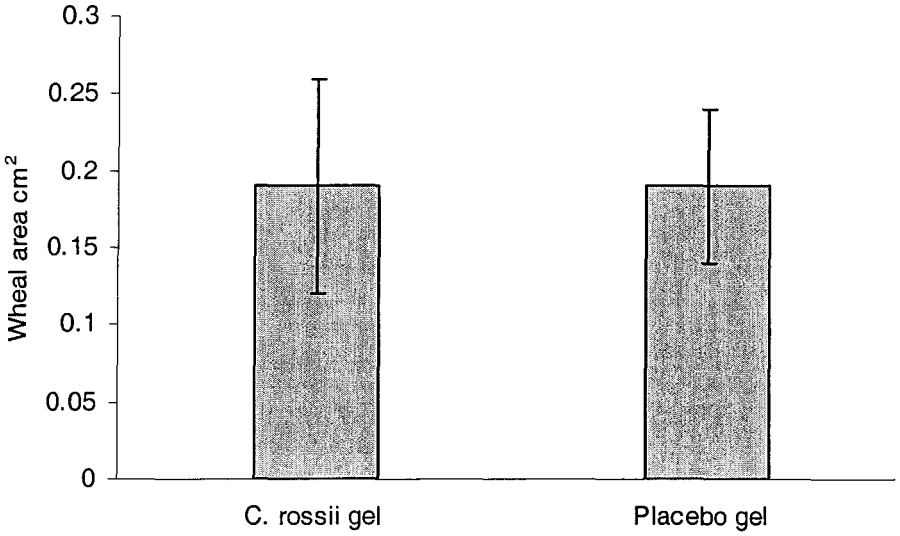


Figure 4.7 Wheal areas in response to the histamine prick test (mean ± SD) after application of *C. rossii* gel or placebo gel.

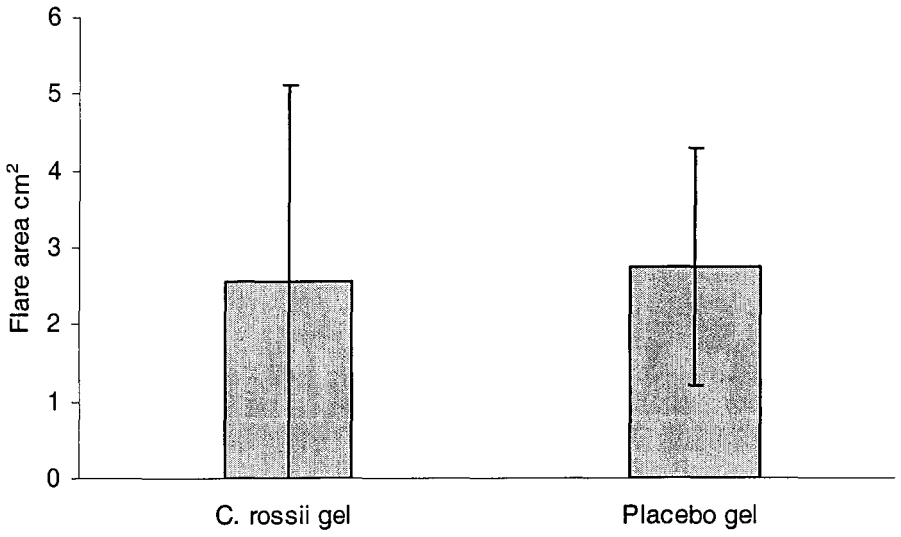


Figure 4.8 Flare areas in response to the histamine prick test (mean ± SD) after application of *C. rossii* gel or placebo gel.

4.1.8 Discussion

The new peaks in the autoclaved sample HPLC chromatogram may be due to the chemical decomposition of sugars or other carbohydrates in the plant juice. These peaks were not observed in freshly prepared gel or gel stored for two months. From the HPLC results it was concluded that there was no significant decomposition of the plant juice in gel on storage for two months, and also that autoclaving of the prepared gel could not be recommended. Prepared gels were therefore used as prepared with no further sterilization. Chlorohexidine used in the gel is protective against microbes and is an effective preservative.

Carpobrotus species have been used as a folk remedy for bites and stings. The fresh plant leaves' juice is normally applied directly to the bite and sting area. *C. rossii* was chosen for the study since it is a Tasmanian native plant, unlike *C. edulis* and *C.*

aequilaterus which were introduced from South Africa. Experimentation was based on anecdotal use of the plant as a folk remedy and *C. rossii* would be the plant most likely used by locals in the past. *C. rossii* had good antioxidant activity among the three *Carpobrotus* species, and it is possible that the agents having antioxidant properties may play a role in the antiinflammatory properties of the plant (Ma and Kinneer, 2002).

The skin prick test is one of the methods used to determine the sensitivity of skin to histamine. It is used to find the ability of a substance or agent to act as an anti-inflammatory agent. The skin prick test was selected to investigate the anti-inflammatory efficacy of a formulation containing *Carpobrotus rossii* plant juice extract.

The histamine skin prick test is easy to perform and the results are straightforward to analyse. The skin prick test has an advantage over other diagnostic test in allergy and attempts to increase the reproducibility are warranted (Malling *et al.*, 1982). The study of histamine has the disadvantage that it accounts for the inflammation produced by histamine only and does not account for the other inflammatory mediators like acetylcholine, bradykinin and prostaglandins. These medications have been shown to produce pain, as well as some of the vascular response characteristic of inflammation (Zachariae and Bjerring, 1990). A positive result in this study would have revealed that the plant has anti-inflammatory action due to inhibition of histamine mediated pathway(s) only, not other mediators' pathways.

There was no statistical difference in the responses to the histamine prick test between the placebo gel treatment and *Carpobrotus rossii* plant juice extract gel treatment. The mean area of wheal after the *C. rossii* (test) gel treatment was 0.19±0.07 cm^2 (mean+SD) and after placebo gel was 0.19±0.05 cm^2 (mean±SD). There were seven subjects, who had a greater area of wheal after placebo than the test gel, but the difference was small and not significant.

The area of flare in placebo gel treated group 2.75±1.74 cm^2 (mean±SD) was not different to the test gel group 2.56±2.56 (mean ± SD). Generally the difference between responses in each arm were small, but there were two subjects that displayed no flare area after treatment with *C. rossii* gel compared with placebo gel treatment.

The mean itching response after *C. rossii* gel was 4.2±1.6 (mean±SD) and after placebo gel was 3.5±2.0 (mean±SD). Five subjects had a higher scale of itching after placebo treatment and 6 subjects had a lower scale of itching after placebo gel, when compared with *C. rossii* gel. One subject had the same scale of itching in both arms.

Wheal reaction did not exhibit any functional relationship with itching intensities in the skin prick test. It has been demonstrated that the skin prick method induced strong and long lasting itching sensations and large flares, but only small wheals (Darsow *et al.*, 1996). In this study there was no relationship between the wheal area and itching intensity and there was no relationship between flare and itching intensity also.

Blood appeared on pricking in 6 subjects on their placebo treatment arm and in eight subjects on their *C. rossii* treatment arm. No subject had bleeding on both arms.

The subjects in the study consisted of 6 males and 6 females with a mean age of 30.7 years (29.8 years for males and 31.5 years for females). It has been shown in previous studies that older age and male sex were associated with a higher response to histamine (Bordignon and Burastero, 2006), but this was not observed in this study. There was no difference between subjects less than 30 years old (mean area of flare was 18.9, wheal was 1.20 and itching response was 23) and subjects more than 30 years old (mean area of flare was 14.1, wheal was 0.74 and itching response was 19).

The results show no evidence of inhibition of the response in humans to the histamine prick test. The results of this study do not exclude anti-inflammatory potential for *C. rossii* plant juice extract, but it can be interpreted that the evidence does not support the case that *C. rossii* extract is active against histamine-induced inflammation.

Stingose® (Pfizer Australia), is a commercially available product for treatment of bites and stings. It is an aqueous solution of 20% aluminium sulphate and 1.1% surfactant that acts by interaction of Al^{3+} ions with proteins and long chain polysaccharide components to inactivate or denature the venoms (Henderson and Easton, 1980). Similarly *C. rossii* plant juice may relieve the pain and discomfort caused by bites and stings, due to its demonstrated content of polyphenolics that may act on proteins and venoms, rather than through any effects on histamine.

A gel was used to incorporate the plant juice extract for use as a topical application. Chlorohexidine Gel APF was used as it is a hydrophilic gel and the plant juice extract is hydrophilic and physically compatible. Tragacanth was used as the gelling agent. It

is a natural product that is compatible with the plant juice extract. Other options of drug delivery were considered including alcoholic solutions, sprays and wipes. The physical stability and compatibility of plant juice extract in an alcoholic solution was not known and it would have been tedious and costly to prepare sprays and wipes. The main drawback of the gel formulation was that there was no means to quantify or control the dose applied. In an attempt to minimize dose variation, the gel was applied only by the principal investigator. However it is probable that some variations in the dose applied did occur. It was assumed that the variations in dose would not be critical enough to influence the results. A further assumption was that the gel would result in good penetration of drug into the skin. The rate of absorption of the potential bioactives from the gel into skin was not measured and was beyond the scope of this investigation. Some of the subjects in the study had no flare after treatment with *C. rossii* gel. This may have been due to sufficient permeability of gel into skin to elicit a response. The rate of dermal penetration was not assessed. A possibility could be the inclusion of skin penetration enhancers if transdermal penetration was found to be a limiting factor.

The study was designed with an assumption that the magnitude and timing of the histamine prick response on the left arm would be the same as the response on the right arm of an individual. But because the timing between the needle prick and measuring the outcome was short, there was some minor variability in measuring the results. It has been demonstrated in a previous study that there was considerable interindividual and intraindividual variability in the response to histamine (Koh *et al.*, 2002).

Apart from the potential involvement of other non-histamine mediators, there are several other factors that may potentially have influenced the responses. Firstly the concentration of the plant juice extract may not have been sufficient to inhibit the histamine-mediated responses. The gel was formulated to have the same concentration of substances as the fresh plant juice. However there may have been some loss of potential actives during formulation of the gel. Increasing the concentration of plant extract in the gel may have changed the responses.

The skin prick test has been found to be dependent on the needle or lancet used to prick the skin. It has been shown previously that a lancet with a 1 mm tip gives the best results. The lancet should be pressed through a drop of histamine test solution at a 90^{o} angle against the skin (Dreborg, 2001). The size of the wheal response is influenced by the composition and potency of the test solution and the pressure applied to the lancet. Histamine concentration can be adjusted, but the pressure is difficult to standardise between different persons and over time, with the same person (Dreborg, 2001). There might be variability in response due to the pricking technique in this study.

The concentration of histamine used was also the minimum concentration used in conventional skin prick tests. A study has suggested that 1 mg/ml histamine dihydrochloride should be replaced by 10 mg/ml of histamine dihydrochloride as the positive reference in routine skin prick tests, in order to get a better reproducibility of wheal reaction. (Taudorf *et al.*, 1985).

Other *in-vitro* methods could be useful in assessing the antiinflammatory activities of *C. rossii* plant juice extract, which do not depend on histamine response alone. Methods such as the Griess bioassay (Dirsch *et al.,* 1998), inhibition of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) (Li *et al.*, 2006) are used in anti-inflammatory assays. These may also prove useful for further evalution of *C. rossii* extract but were beyond the scope of the current study.

From the results of the study it was shown that there was no evidence of overall anti-inflammatory activity of *Carpobrotus rossii* plant juice extract formulated in a gel against histamine.

4.2 Anti – wart clinical trial

4.2.1 Objectives

To compare the efficacy of a pharmaceutical gel containing *Carpobrotus rossii* plant juice extract against placebo gel in the treatment of non-genital, flat warts in adults.

4.2.2 Introduction

Warts are small, benign growths caused by a viral infection of the epidermis (Sterling *et al.*, 2001). The virus causing warts belongs to the human papilloma virus (HPV) family (Sterling *et al.*, 2001). Viral warts are a common dermatological condition. Although the rate of spontaneous recovery is high, it usually takes a long time (Al-Gurairi *et al.*, 2002). Warts usually appear in children and young adults and are usually not cancerous. One of the reasons for their appearance in children is lack of immunological resistance and low immune power (Brodell and Johnson, 2003). Warts appear as a small lump of hard skin with black dots and are usually painless. The more common types of warts include: common hand warts, foot warts and genital warts. The major ways of treating warts presently are: cryotherapy; topical chemicals, including acids; surgical removal and immune modulators or immune promoters (Brodell and Johnson, 2003). The immune response modifiers include imiquimod and resiqimod. Imiquimod is a cytokine inducer and a modifier of the innate immune response as well as acquired antiviral and antitumor immune responses (Vidal, 2006).

Anecdotally *Carpobrotus* plant juice is a folk remedy for treating warts. Oxalic acid, catechins, some flavonoids and carbohydrates were identified in *Carpobrotus edulis*

plant juice (Vennavaram, 2005). Salicylic acid is used as one of the important treatments for warts but side effects with salicylic acid therapy include irritation, redness and injury to surrounding tissue (Lebwohl, 1999). Like imiquimod, *Carpobrotus* species may possess compounds with an immunomodulation mechanism of action in treating warts without the side effects of salicylic acid. It was therefore appropriate to conduct a scientific trial on the effectiveness of *Carpobrotus* plant juice in the treatment of cutaneous warts (flat warts).

4.2.3 Methods

4.2.3.1 Study design

This study was a placebo controlled (double blinded) trial for the treatment of non–genital, flat warts using a topical pharmaceutical gel containing *Carpobrotus rossii* plant juice extract and a placebo gel. Participants were human subjects who were above 18 years of age having one or more warts affecting their hands. The primary outcome measure(s) were healing of the warts and the method of assessment was by subject's self-assessment of wart healing.

4.2.3.2 Gel preparation and Randomisation

The pharmaceutical gel formulations were prepared at the School of Pharmacy as described in section 4.1.5.2 of this thesis. Randomisation was performed by a person who was not otherwise involved in the research. Radomisation was done using a computer-generated list of random numbers. Gels, either *C. rossii* or placebo, that were identically labelled, were given a consecutive numerical subject code on the

basis of the randomisation schedule. Subjects were given a subject code upon enrollment that was used to allocate their gel, either placebo or *C. rossii.*

4.2.3.3 Study protocol

Recruitment was carried out by using posters and notices displayed at the university campus and by placing advertisment in newspapers to notify potential participants about the study (Appendix 1).

Exclusion criteria for subjects are shown in Table 4.3.

Table 4.3 Exclusion criteria for potential subjects for the wart trial.

Subjects below the age of 18 years
Warts with hair
Inflamed warts
Immunosuppressed patients
Subjects with other skin diseases
Subjects with warts treated with other medication or therapy
Pregnant and lactating women

On enrolling, subject's personal data (sex, age, first name), case history (skin allergies, other wart medications) were recorded. The subject was examined for the number of warts on each hand. The warts were mapped by the subjects themselves. An image of the subject's hand was obtained by tracing around the hand on plain A4 size paper with the help of a pen. Onto this image the approximate size and location of each wart was recorded. Then the paper was photocopied. The original sheet was kept with the investigator and the photocopied sheet was given to the subject to assist with weekly self assessment.

Subjects were advised to apply the gel four times a day on the warts for a period of 8 weeks unless warts disappeared within eight weeks. Subjects were asked to do a weekly examination of hands having warts, to observe the healing of warts and also to note the arrival of any new warts, and to record any changes on their wart map. Subjects were supplied with a case diary to record any adverse effects and to note the date when wart healing occurred. In the case of an adverse reactions, a physician from the University of Tasmania Clinical School was available to consult with the affected subject.

4.2.3.3 Method of analysing results

The study was completed when all the completion forms and self assessment results were collected from the subjects. The data from the subject self assessment sheets was recorded and the unblinding of randomization was done. The demographic features of the control and treatment groups were compared statistically. The cohort age of subjects was compared using Students' t-test. Cohort gender was compared using the Chi-square test. The wart index was calculated for each subject by dividing the number of warts healed by the initial number of warts present and multiplying by 100. Comparisons between responses in treatment and control groups were performed using the Mann-Whitney U test.

4.2.4 Results

Subjects recruited in this study numbered 50 and among them 41 subjects completed the study with a dropout of 9 subjects. There was no evidence of the overall anti-wart activity of the *Carpobrotus rossii* plant juice extract gel.

Table 4.4 Demographic data of subjects enrolled in the wart study with statistical comparisons between *Carpobrotus* gel treatment and placebo groups.

	C. rossii gel (Treatment group)	Placebo gel (Control group)	
Age (mean±SD)	39.9±16.1 (n=19)	36.2±16.8 (n=22)	t=0.657, df=33, p=0.52
Sex (male/female)	11/8	10/12	χ^2=0.63, df=1, p=0.54
Number of warts median, (range)	2 (1-29), (n=19)	3 (1-72), (n=22)	Mann-Whitney U=205.0, z=-0.105, p=0.92

Table 4.5 Wart index and cure rate of warts treated with placebo or *C. rossii* gel

	Carpobrotus gel	Placebo gel	p-value
Wart index	5.5	11.4	0.73
Cure rate	1.6 % (2/127)	3.4% (6/178)	0.48

There were no differences in age (p=0.52), sex (p=0.54) and number of warts (p=0.92) between the treatment group and the control group, as shown in Table 4.4.

Figure 4.9 shows the frequency distribution of the number of warts in each subject in the *C. rossii* gel treatment group and Figure 4.10 shows the frequency distribution of subjects in the placebo gel treatment group.

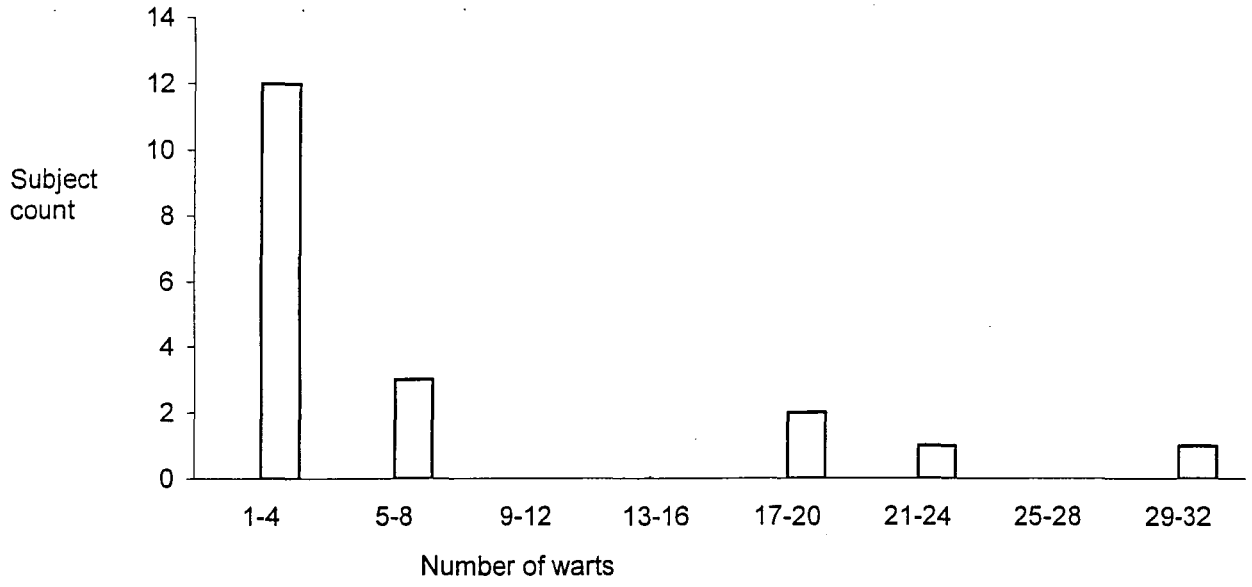


Figure 4.8 Frequency distribution of warts in subjects treated with *C.rossii* gel (active)

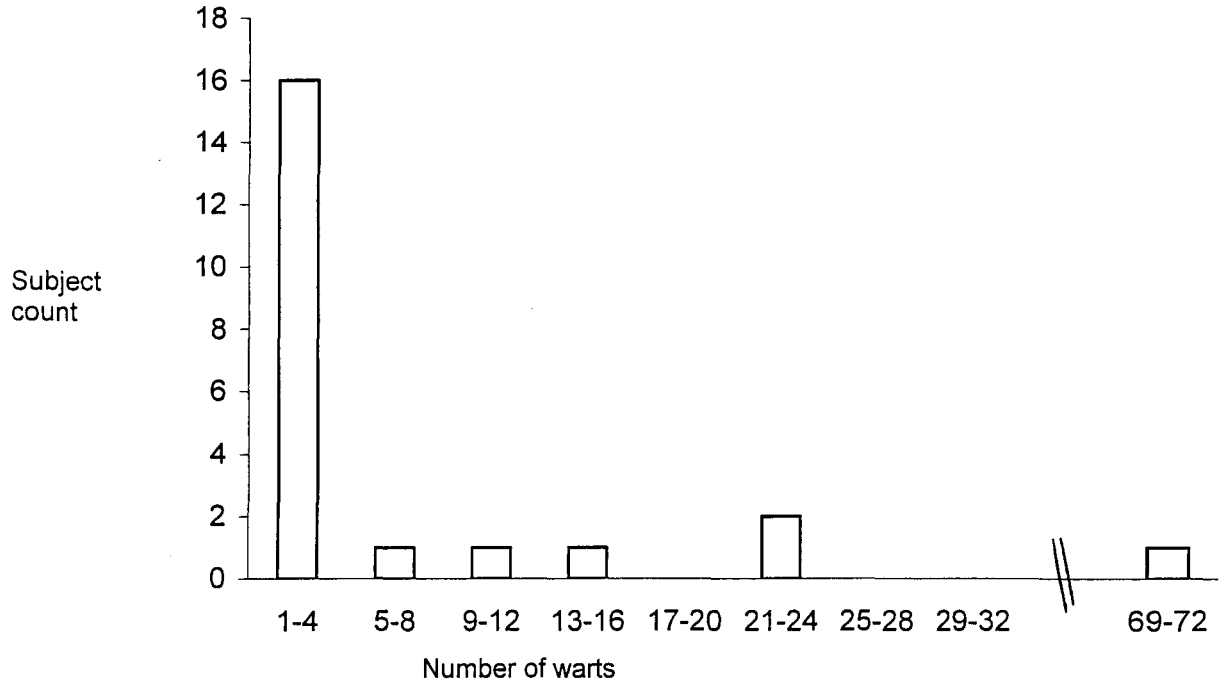


Figure 4.9 Frequency distribution of warts in subjects treated with placebo gel

A total of 305 warts were studied. Analysis of cure rate based on individual warts revealed no difference between *C. rossii* and placebo treatments (χ^2=0.94, df=1, p=0.48). As summarised in Table 4.5 the cure rate was 1.6% (2/127) for *C. rossii* gel treatment and 3.4% (6/178) for placebo gel with an odds ratio of 0.46 [95%CI; 0.09-2.31]. A single patient with 72 warts in the control group was excluded for a secondary analysis which again revealed no difference between *C. rossii* and placebo treatments (χ^2=2.91, df=1, p=0.15) and an increase in cure rate to 5.7% for the controls.

Analysis of efficacy based on individual patients using a wart index score out of 100, based on (completion warts)/ (baseline warts) x100 again showed little efficacy with either treatment arm or difference between groups. Mean scores were similar (5.5 versus 11.4) for *C. rossii* and placebo treatments respectively (Mann-Whitney U=201.5, z=-0.196, p=0.73) with a range from 0 (not effective) to 100 (all warts cured). One patient reported six new warts during the eight week study period which were excluded from the analysis. Excluding the patient with 72 warts resulted in little change with subsequent mean scores similar (5.5 versus 11.9) for *C. rossii* and placebo treatments respectively (Mann-Whitney U=191, z=-0.230, p=0.69).

4.2.5 Discussion

The results of this study show no significant difference in activity against warts between placebo gel and *C. rossii* treatment gel.

Carpobrotus species have been used as a folk remedy for the treatment of warts. The fresh plant leaf juice was normally applied directly to the warts. The result of this

study does not support the traditional use as wart treatment but it does not completely exclude the plant from its anti-wart activity. It is possible that preparation of the formulation might have altered the chemistry of the plant juice constituents. Stability studies were performed on the gel, which showed no change in the composition of the gel after two months. However changes may have occurred during gel preparation. Also, the stability of the gel was analysed only for the changes in the carbohydrates and flavonoids, not for any changes in the acid fraction of the plant juice. The plant juice was acidic and contained oxalic acid and other acidic constituents. Many topical applications used for the treatment of warts contain salicylic acid, formic acid and trichloroacetic acid as active ingredients. The mechanism of action of salicylic acid in elimination of warts involves keratolysis of virally infected tissue. Trichloroacetic acid and bichloroacetic acid are powerful irritants that work by hydrolysing the cellular proteins leading to inflammation and cell death (Bhat *et al.*, 2001). The *C. rossii* formulation may not have had as high a concentration of the acids or other active constituents as the plant juice. It may need a greater concentration in the gel preparation in order to exert anti-wart activity.

Another natural remedy for the treatment of warts is the radium plant milk, *Euphorbia peplus*. The milky sap of this plant is used as a home treatment for warts and basal cell carcinomas (Weedon and Chick, 1976). The major constituents of *E. peplus* are chlorogenic acid and flavonoids like quercetin, kaempferol and ferulic acid (Nagwa *et al.*, 2002). *Carpobrotus* species also have flavonoids as major chemical components.

Recalcitrant viral warts are one of the most common therapeutic problems. These have been unsuccessfully treated with paring, keratolytic and cryotherapy and may need a variety of additional treatments such as intralesional bleomycin, topical

retinoids, laser destruction and oral cimitedine (Buckley *et al.*, 1999). In our trial we didn't account for the recalcitrant warts which are hard to cure with single treatment. Immunotherapy with the universal allergic contact sensitizer diphencyprone is an effective option for the treatment of recalcitrant viral warts (Buckley *et al.*, 1999). *Carpobrotus* species plant juice has no contact sensitizer as such and might not be able to cure warts by such a mechanism.

Combinational therapy is always more effective than a single therapy. The combination of 5-flurouracil and salicylic acid is an effective and beneficial therapy for common and plantar warts (Zschocke *et al.*, 2004). An ointment comprising 0.1% diphencyprone and 15% salicylic acid in white soft paraffin wax is effective in treatment of palmoplantar warts (Armour and Orchard, 2006). So a combination of the *Carpobrotus* species plant juice extract with other agents like salicylic acid may be effective in treatment of warts.

Based on this trial, *C. rossii* is not an effective anti-wart treatment and offers no potential for further anti-wart investigations. *C. edulis* and *C. aequilaterus* have different chemistry from *C. rossii.* All the species contain flavonoids but those from *C. edulis* and *C. aequilaterus* are quite different from those from *C. rossii.* It is a remote possibility that *C. edulis* or *C. aequilaterus* may be effective in treatment of warts.

Conclusion

The chemical composition of the plants *Carpobrotus aequilaterus, C. edulis* and *C. rossii,* (Aizoaceae) have been investigated. The plant juice consisted mostly of flavonoids, tannins and carbohydrates. *C. edulis* and *C. aequilaterus* contained three flavonoids in common, none of which have been reported from *Carpobrotus* species previously. *C. rossii* contained four novel flavonoid that were substituted with a moiety consistent with 3-hydroxy-3-methylglutaric acid. Further structural characterisation of these flavonoids and screening for their biological activities is recommended. The antioxidant activity of *Carpobrotus* species is significantly potent and a further investigation of the antioxidant compounds and their potential therapeutic applications warrant investigation. Antioxidant activity guided fractionation by gel filtration column chromatography and analysis of this fraction by LC-MS showed the presence of condensed tannins as major chemical constituents, which appeared to account for the majority of the antioxidant activity. *C. rossii* extract formulated as a gel was not effective in the treatment of common warts and did not possess topical anti-inflammatory action against histamine.

In spite of the negative results from the clinical trials conducted in this study, a potent antioxidant activity has been identified and novel flavonoids have been detected. Further investigations of the pharmaceutical potential for *Carpobrotus* species are warranted.

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Appendix

The advertisement for the clinical trails on warts was given in daily local newspaper "The Mercury". The notices and posters were pasted at notices boards in all the Schools of University of Tasmania; they were also pasted in Clinical School, University of Tasmania. Subjects' self assessment form and subject case sheet were given on enrolling into the study.

On the self assessment form a hand imprint of the patient was taken along with the approximate location and size of the wart .

Subject name..... contact name..... study code.....

Details of wart self assessment

Date of weekly self assessment		Warts no longer present(Wart number)	New warts present (mark on diagram) with date and new number.
week			
1			
2			
3			
4			
5			
6			
7			

8			

subject case sheet

Record of gel application to the warts - please tick yes or no

	Monday		Tuesday		Wednesday		Thursday		Friday		Saturday		Sunday	
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
morning	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
afternoon	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
evening	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
night	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no